Biotechnological approaches for sugarcane enhancement – Drought tolerance and Sucrose accumulation

by

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Declaration

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Abstract

The application of biotechnological approaches can supplement traditional crop breeding strategies to combat the effects of adverse environmental conditions on plant production in order to bridge the gap between supply and demand worldwide. In Southern Africa adverse environmental conditions, such as drought, are a serious agronomic problem that threatens crop productivity, food security and eventually negatively impact the economy of the region. Urgent attention to overcome drought stress is therefore vital to eradicate or minimise its effects on crop productivity. Furthermore, the use of fossil fuels as the primary source of energy worldwide has led to demonstrable detrimental effects to the environment and its sustainability. Efforts have been made to seek other cost effective energy sources that counteract the disadvantages of fossil fuels towards the environment. In this regard, research has unravelled the potential of feedstock such as maize and sugarcane as fuel sources and tremendous advancements have been made to explore the possibility of using both sugarcane and maize for biofuel production.

Sugarcane is an economically important crop that can be exploited both as food source and for production of biofuels. This study aimed to apply biotechnological methods to: firstly, produce drought tolerant sugarcane plants from three drought sensitive cultivars of South African origin using mutation breeding; secondly, manipulate the *myo*-inositol oxidation pathway (MIOP) in the model NCo310 sugarcane cultivar to alter sucrose and possibly cell wall composition which would enhance the plants viability for second-generation biofuel production.

To accomplish this, optimal *in vitro* growth conditions for callus obtained from NCo376, N19 and N40 sugarcane cultivars were first established by testing different auxin concentrations for *in vitro* callus induction. NCo376, N19 and N40 callus grew optimally on 1, 0.5 and 2 mg/L 2,4-D, respectively. The NCo310 model cultivar was furthermore exposed to different ethylmethanesulfonate (EMS) concentrations to induce random mutations without compromising *in vitro* plant regeneration abilities of the model cultivar. Subsequently an optimal EMS concentration of 16 mM was selected and used for all further mutagenesis experiments across all cultivars. Additionally, calli from the NCo310 cultivar were exposed to polyethylene glycol (PEG) and mannitol at different concentrations to identify a suitable osmoticum, which simulates a drought stress environment *in vitro*. PEG at a 20% (w/v) concentration was determined as the most suitable osmoticum to induce negative osmotic pressure and allow selection of drought tolerant sugarcane plantlets *in vitro*. Sugarcane cultivars included in this study were consequently exposed to 16 mM EMS and *in vitro*

selected on 20% PEG, which resulted in the survival of 7, 18 and 19 plantlets of NCo376, N19 and NCo310, respectively. These plantlets were multiplied to prepare for future *ex vitro* drought pot trials. NCo310 *in vitro* selected lines were subjected to *ex vitro* preliminary and follow-up pot trials to confirm drought tolerance. In the preliminary drought trials, single biological clones of selected lines were exposed to water deficit conditions. Of the 19 plantlets selected *in vitro*, only five outlived the wildtype NCo310 control plants. Plants were re-watered three days after plant to monitor possible plant recovery. Only one line, 310EP 4.1A, recovered from the drought stress and formed new shoots upon re-watering. To confirm these preliminary results, follow-up drought pot trials were executed using three biological repeats of each line together with NCo310 wildtype controls. . Preliminary results from this study indicate that biotechnological approaches, such as mutation breeding and *in vitro* selection for osmotic pressure, can potentially be successfully applied to develop crops to combat the negative effects of abiotic stresses, such as drought.

Nucleotide sugars are activated monosaccharides that act as glycosyl donors in reactions that contribute to the production of cell wall biopolymers in plants. Cell wall components might play a role in biofuel potential of crops and are synthesised by various pathways. The nucleotide 5'-diphospho-sugar (NDP-sugar) interconversion pathway uses the sequential action of kinases and NDP-sugar phosphorylases to produce uridine 5'-diphosphate glucuronic acid (UDP-glucuronic acid), a precursor for cell wall sugars and lignocellulosic polymers. The NDP-sugar interconversion pathway is composed of the sugar nucleotide oxidation pathway (SNOP) and the *myo*-inositol oxidation pathway (MIOP). In this study one of the MIOP enzymes isolated from *Arabidopsis thaliana*, the glucuronokinase enzyme was overexpressed in NCo310 sugarcane, to evaluate changes in sucrose content. Morphological differences were observed between transgenic lines and the NCo310 wildtype, no significant sucrose content or glucoronokinase enzymatic activity differences were detected in leaf material isolated from these lines. Inconclusive results obtained from this study, together with the lack of published literature on the production of cell wall components and still remaining knowledge gaps regarding sucrose metabolism by the NDP-sugar interconversion pathways in sugarcane, demonstrate the need for further research into this particular field of cell wall biosynthesis and sucrose metabolism.

Opsomming

Tradisionele gewastelingstrategieë kan aangevul word deur die toepassing van biotegnologiese benaderings, om sodoende die gevolge van ongunstige omgewingstoestande op plantproduksie teë te werk en die wêreldwye gaping tussen vraag en aanbod te oorbrug. In Suidelike Afrika is ongunstige omgewingstoestande, soos droogte, 'n ernstige agronomiese probleem wat gewasproduktiwiteit en voedselsekerheid bedreig en uiteindelik 'n negatiewe uitwerking op die streek se ekonomie het. Dringende aandag aan die oorkoming van droogtestres is daarom noodsaaklik om die uitwerking daarvan op gewasproduktiwiteit te neutraliseer of te verminder. Boonop het die wêreldwye gebruik van fossielbrandstowwe as hoofenergiebron tot bewysbare skadelike gevolge vir die omgewing en die volhoubaarheid daarvan gelei. Daar word tans gepoog om ander kostedoeltreffende energiebronne te vind, wat die nadele van fossielbrandstowwe vir die omgewing sal teëwerk. In dié verband het navorsing die potensiaal van roumateriale soos mielies en suikerriet as moontlike brandstofhulpbronne blootgelê, en groot vooruitgang is reeds gemaak om die moontlikheid van hierdie gewasse vir biobrandstofvervaardiging te ondersoek.

Suikerriet is 'n ekonomies belangrike gewas wat benut kan word met die oog op die verligting van voedselvoorraadtekorte en die vervaardiging van biobrandstowwe. Hierdie studie is gemik op die toepassing van biotegnologiese metodes om: in die eerste plek droogtebestande suikerrietplante uit drie droogte-sensitiewe kultivars van Suid-Afrikaanse oorsprong aan die hand van mutasieteling te vervaardig; en in die tweede plek die *mio*ïnositol-oksidasieroete (MIOP) in die model NCo310-suikerrietkultivar te manipuleer ten einde sukrose, en moontlik selwandinhoud, te wysig en daardeur die plante se kiemkrag vir tweedegenerasie-biobrandstofvervaardiging te verhoog.

Om dit te kon vermag, moes daar eers optimale *in vitro*-groeitoestande vir *Saccharum*-spesiehibriedkultivars NCo376, N19 en N40 bepaal word deur verskillende ouksienkonsentrasies vir *in vitro*-kallusinduksie te toets. NCo376, N19 en N40 het optimaal op onderskeidelik 1, 0.5 en 2 mg/L 2,4-D gegroei. Die NCo310-modelkultivar is verder aan verskillende konsentrasies etielmetaansulfonaat (EMS) blootgestel om willekeurige mutasies te induseer teen 'n koers wat waarskynlik 'n hoë mutasiefrekwensies tot gevolg gehad het, sonder om *in vitro*-plantregenerasievermoëns te kompromitteer. Daarna is 'n optimale EMS-konsentrasie van 16 mM geselekteer en vir alle verdere mutagenese-eksperimente oor al die kultivars heen gebruik. Verder is kallusse van die NCo310-kultivar aan verskillende

konsentrasies poliëtileenglikol (PEG) en mannitol blootgestel om 'n geskikte osmotikum te identifiseer wat 'n droogtestresomgewing *in vitro* simuleer. PEG, teen 'n konsentrasie van 20% (w/v), is bepaal as die mees geskikte osmotikum om negatiewe osmotiese druk te induseer en die seleksie van moontlike droogtebestande suikerrietplantjies *in vitro* moontlik te maak. Die suikerrietkultivars wat by hierdie studie ingesluit is, is daarna aan 16 mM EMS blootgestel en *in vitro* op 20% PEG geselekteer, wat tot die oorlewing van 7, 18 en 19 plantjies onderskeidelik van NCo376, N19 en NCo310 gelei het. Hierdie plantjies is verdeel om vir toekomstige *ex vitro*-droogteproewe in potte voor te berei. NCo310-, *in vitro*-geselekteerde lyne is aan voorlopige *ex vitro*- en opvolgproewe onderwerp om droogtebestandheid te bevestig. In die voorlopige droogteproewe is enkel- biologiese klone van geselekteerde lyne aan watertekorttoestande blootgestel. Van die 19 plantjies wat *in vitro* geselekteer is, het slegs 5 die wildetipe-NCo310 kontroleplante oorleef. Plante is weer drie dae ná plantsterfte nat gegooi om moontlike plantherstel te monitor. Slegs een lyn, naamlik 310EP 4.1A, het van die droogtestres herstel en nuwe lote uitgestoot nadat dit weer nat gegooi is. Om hierdie voorlopige resultate te bevestig, is opvolgdroogte-potproewe met behulp van drie biologiese herhalings van elke lyn uitgevoer, ingesluit 310EP 4.1A, tesame met die NCo310 wildetipe-kontroles. Alle lyne, behalwe 310EP 4.1A, het die kontroleplante oorleef. Nadat dit weer nat gegooi is, het slegs lyn 310EP 4.1A van die geïnduseerde droogtestrestoestande herstel. Die voorlopige resultate van hierdie studie dui gevolglik daarop dat biotegniese benaderings, soos mutasieteling en *in vitro*-seleksie vir osmotiese druk, moontlik suksesvol toegepas kan word om gewasse te ontwikkel wat die negatiewe gevolge van abiotiese strestoestande soos droogte kan weerstaan.

Nukleotiedsuikers is geaktiveerde monosakkariede en vervul die rol van glikosielskenkers by reaksies wat tot die vervaardiging van selwandbiopolimere in plante bydra. Selwandkomponente speel 'n deurslaggewende rol by die biobrandstofpotensiaal van enige gewas en word via verskeie roetes gesintetiseer. Die onderlinge omsettingsroete van nukleotied 5'-difosfo(NDP)-suiker gebruik die opvolgaksie kinase en NDP-suikerfosforilase om uridien 5'-difosfaat(UDP)-glukuroniesuur te vervaardig, 'n voorloper vir selwandsuikers en lignosellulosepolimere. Die onderlinge omsettingsroete van NDP-suiker sluit die suikernukleotiedoksidasieroete (SNOP) en die *mio*ïnositoloksidasieroete (MIOP) in. In hierdie studie is een van die MIOP-ensieme wat uit *Arabidopsis thaliana* geïsoleer is gevolglik ooruitgedruk in NCo310-suikerriet, ten einde die veranderings in sukrose-inhoud te evalueer. Alhoewel morfologiese verskille tussen transgeniese lyne waargeneem is toe die glukoronikase-geen in die NCo310-wildetipe ooruitgedruk is, is geen beduidende verskille in sukrose-inhoud of glukoronikase- ensimatiese aktiwiteit bespeur in blaarmateriaal wat uit hierdie lyne geïsoleer is nie. Die onbesliste resultate wat uit hierdie studie bekom is, die

gebrek aan gepubliseerde literatuur oor die vervaardiging van selwandkomponente, asook die bestaande kennisgapings betreffende sukrosemetabolisme by die onderlinge omsettingroetes van NDP-suiker in suikerriet, dui op die behoefte aan verdere navorsing in hierdie spesifieke veld van selwandbiosintese en sukrosemetabolisme.

Thesis Outline

This thesis is divided into the following chapters:

Chapter 1:   The first chapter is comprised of a literature overview on sugarcane, its importance and potential improvement to overcome environmental and economic challenges. This chapter discusses sugarcane as a globally important crop and the application of traditional breeding and its limitations in crops with complex genomes such as sugarcane. It further discusses modern techniques to assist in improving sugarcane. Additionally the chapter addresses two specific issues relevant to the South African sugarcane industry. These issues include the viability of sugarcane as a biofuel crop as well as the effect of agronomic drought to the South African sugar industry.

Chapter 2:   The chapter focuses on agronomic drought and its effect on sugarcane and attempt to improve the drought tolerance of some susceptible sugarcane cultivars. It is comprised of an introduction, materials and methods, results and discussion of mutagenic manipulation. This is followed by *in vitro* osmotic selection and *ex vitro* drought pot trials to confirm drought tolerance in selected mutant plantlets.

Chapter 3:   This chapter focuses on the viability of sugarcane as a biofuel crop and it consists of an introduction, materials and methods, results and discussion on genetic manipulation of sugarcane by overexpression of a glucuronokinase gene to observe its effect on sucrose accumulation for biofuel production.

Chapter 4:   A general summary and future recommendations based on results obtained from chapters 2 and 3.

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I dedicate this work to my late mother, the woman who pursued her children against all odds. May your soul rest in peace Letebele, re tla lula re hopola lerato la hao ka mehla.

List of Abbreviations

°C	Degree Celsius
%	Percentage
µM	Micromolar
µmol	Micromole
µm	Micrometer
2,4 D	2,4 Dichlorophenoxyacetic acid
310EP	NCo310 EMS and PEG
ATP	Adenosine triphosphate
bp	Base pair (s)
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium chloride
CaMV 35S	Cauliflower mosaic virus 35 S
cDNA	Complementary DNA
cm	Centimetre (s)
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetra acetic acid
EMS	Ethylmethane sulfonate
F6P	Fructose-6-phosphate
FW	Fresh weight
g	grams
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
g/l	Gram per litre
gDNA	Genomic DNA
GMO	Genetically modified organism
HK	Hexokinase
Inv	Invertase
KCl	Potassium chloride

Kg	Kilograms
KOH	Potassium hydroxide
kPa	kilopascals
L	Liter
LB	Luria Bertani
LDH	Lactate dehydrogenase
mgCl$_2$	Magnesium chloride
mm	Millimeters
M1	First generation of mutated plants
MIOP	*Myo*-Inositol oxidation pathway
MIOX	*Myo*-Inositol oxygenase
MS	Murashige and Skoog medium
NAA	1-Naphthaleneacetic acid
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide reduced
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NADP	Nicotinamide adenine dinucleotide phosphate
NDP	Nucleotide 5'-diphosphate
npt II	Neomycin phosphotransferase II
OD	Optical density
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEP	Phosphoenol pyruvate
PGI	Phosphoglucose isomerase
PK	Pyruvate kinase
PMSF	Phenyl methyl sulfonyl fluoride
RNA	Ribonucleic acid
RPM	Revolution per minute
SMC	Soil moisture content
SNOP	Sugar nucleotide oxidation pathway
SPS	Sucrose phosphate synthase
SqRT-PCR	Semi quantitative Real Time-Polymerase chain reaction
SuSy	Sucrose synthase
TBE	Tris-Boric acid-EDTA buffer
U	Enzyme unit

Ubi 1	Maize ubiquitin promoter
UDP	Uridine 5'-diphosphate
UGD	UDP-Glucose dehydrogenase
USP	UDP-Sugar pyrophosphorylase
UV	Ultraviolet
v/v	Number of mL per 100 mL concentration expression
w/v	Number of grams per 100 mL concentration expression
WT	Wild type

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Chapter 1

Sugarcane, its importance and improvement to overcome environmental challenges

1.1 Introduction

1 1.1Sugarcane – an important global crop

Sugarcane (*Saccharum* spp.) is a highly productive C4 metabolic perennial crop and a member of the Poaceae family, an economically valuable grass family that includes crops such as maize, rice, wheat and sorghum (Edwards and Huber, 2014; Zingaretti *et al.*, 2012). It is a globally important source of sucrose and ethanol, and accounts for over 75% of the world's total sugar production (Baucum *et al.*, 2009). It is cultivated in 120 countries on a total of 23.81 million hectares of land, with an annual worldwide harvest of 1.685 billion tons (Lam *et al.*, 2015). According to The Statistics Portal, the leading sugarcane producers worldwide in 2013 (http://www.statista.com/statistics/267865/principal-sugar-cane-producers-world wide/) included Brazil, India, China, Thailand and Pakistan. In Brazil alone, more than 9 million hectares of land was used for cultivation of sugarcane in 2012 (Zingaretti *et al.*, 2012). South Africa is the 15th largest sugarcane producer in the world. The South African sugarcane industry uses approximately 380 000 hectares of land, producing around 18 million tons of sugarcane compared to Brazil's 739.27 million tons per year.

Sugarcane is optimally cultivated in tropical and sub-tropical regions (Zingaretti *et al.*, 2012). Its growth and development is sensitive to numerous agronomic and environmental factors such as, unstable climate conditions, soil types, pests and diseases, interference of weeds and water availability (Galon *et al.*, 2013; Cardozo and Sentelhas, 2013; Singh *et al.*, 2013; Qureshi and Afghan, 2005). It is therefore evident that its productivity is dependent on application of intensive knowledge and proper management strategies to its cultivation. However, due to increasing sugar and ethanol demands, predictions estimate about 64 million hectares of arable land would be required to bridge the sugarcane supply and demand gap in less than five years from now (Zingaretti *et al.*, 2012; Hoogwijk *et al.*, 2003; Régis *et al.*, 2010).

1.1.2 Traditional breeding of sugarcane and its limitations

With the increasing demand for sugarcane, factors such as arable land area and water availability consequently become of crucial concern in terms of meeting demands for sugarcane production, especially when the crop's adaptation is primarily achieved through traditional breeding. Through traditional breeding, development of new and desirable crop traits can take 12 to 25 years to accomplish, especially for traits that are difficult to manipulate (Selvi and Nair, 2010; Borlaug, 1983; Ashraf *et al.*, 2008; Flowers, 2004; Yamaguchi and Blumwald, 2005). For example, while some traits, like brown rust resistance, may be controlled by a single gene (*Bru1*), complex traits such as resistance to abiotic stresses and/or pests and diseases are controlled by multiple genes, therefore impeding the achievement of desired traits in a short time span (Racedo *et al.*, 2013; Parco *et al.*, 2014; Manigbas and Villegas, 2007; Jain, 2005; Suprasanna *et al.*, 2012; Devarumath *et al.*, 2008). Despite the increasing interest in sugarcane because of its commercial demand, the crop's genomic complexity and large genome size hinders the progression of new cultivars through traditional breeding. Most sugarcane cultivars used for commercial purposes are highly polyploid and aneuploid hybrids of a cross between *S. officinarum* and *S. spontaneum*. These cultivars are characterised by vigour and resistance genes from the wild *S. spontaneum* as well as high sucrose content from the *S. officinarum* (Grivet and Arruda, 2001). Genomic *in situ h*ybridisation (GISH) has shown that these hybrids contain about 10 – 20% *S. spontaneum,* 80 – 90% *S. officinarum* and a small percentage of recombinant genomic contributions between the two species (Piperidis *et al.*, 2010; D'Hont, 2005).

Sugarcane hybrids have chromosome numbers varying from 100-130, with a genome size of 10 000 mega base pairs (Mb) and a diploid number (2n) of 115, which is much larger than that of maize (5 500 Mb; 2n = 20), rice (860 Mb; 2n = 24) or sorghum (1 600 Mb; 2n = 20) (D'Hont and Glaszmann, 2001). Furthermore, traditional sugarcane breeding is often restricted due to the lack of pollen or pollen infertility as a result of unfavourable climatic conditions (Brett, 1950). Moreover, it is a general belief that the genetic diversity within the sugarcane gene pool has reached a threshold and will not allow for additional introduction of favourable traits through traditional breeding (Mariotti, 2001). Alternative breeding or technological approaches are therefore needed to introduce new genetic varieties in order to improve or stabilise sugarcane's productivity and yield to meet the crop's growing commercial demand.

1.1.3 Modern techniques applied to assist in improving sugarcane

Biotechnological techniques such as genetic transformation, marker-assisted selection and induced mutagenesis, among others, have shown tremendous promise in assisting plant breeders and researchers to achieve desirable traits in plants regardless of genome complexity. Because these techniques are able to directly target genes of interest, they reduce difficulties and time required to produce new and/or desired traits. In addition, they provide the possibility of screening for traits that are governed by numerous genes such as resistance or tolerance to abiotic stresses, pests and diseases and they also make it possible to screen for numerous traits simultaneously (Manigbas and Villegas, 2007).

As previously mentioned, sugarcane hybrids are comprised of a complex genome, for which the genome sequence data is still not available. Knowledge of plant genomes, such as those of sorghum and *Arabidopsis thaliana*, which have been fully sequenced and annotated have made sugarcane genomic studies possible over the past 15 years (de Setta *et al.*, 2014). Because of the similarities between the sorghum and sugarcane genomes, both members of the Poaceae grass family, the sorghum genome has been used as a reference genome for sugarcane genomic studies using biotechnological approaches such as plant genetic engineering. Sugarcane studies have resulted in the development of different sugarcane cultivars with desired traits (Koch *et* al., 2012; Sandhu *et al.*, 2002; Gallo-Meagher and Irvine, 1996; Falco *et al.*, 2000; Leibbrant and Snyman, 2003). For example, a *cry1*A (cryptochrome circadian clock 1A) gene was introduced, conferring insect resistance (Rapulana and Bouwer, 2013). A synthetic CP4 *epsps* (5-enolpyruvulshikimate-3-phosphate synthase from *Agrobacterium tumefaciens* CP4 strain) gene from *Arabidopsis* was introduced, enabling glyphosate tolerance and conferring herbicide tolerance in this crop (Noguera, *et al.,* 2015). Sugarcane plants now also contain transgenes to improve resistance to sugarcane mosaic virus, (Falco *et al.*, 2000), cane grubs (Allsopp and Suasa-ard, 2000; Nutt *et al.*, 2001), chlorotic symptom induction, and multiplication and systematic invasion by either *Xanthomonas albilineans* (Zhang *et al.*, 1999) or Fiji disease (McQualter *et al.*, 2004). Nevertheless, despite a large number of genetic engineering research studies, so far only one commercial GM sugarcane variety has been released, a drought tolerant variety in Indonesia in 2013 (Noguera, *et al.,* 2015).

Overall, genetic engineering for increased sucrose and yield in sugarcane has been difficult and met with limited success (Wu and Birch, 2007). Elite cultivars demonstrate slight increase in sucrose levels when single sugarcane genes were manipulated. Transgenic studies have, for example manipulated activities of several enzymes in attempts to increase sucrose levels in sugarcane. These include, reducing the PFP (D-fructose-6-phosphate 1-

phosphotransferase) activity in sugarcane young and mature internodes, which resulted in changes in sugar cycling (Groenewald and Botha, 2008; van der Merwe *et al.*, 2010) and transforming sugarcane with sucrose 1-fructosyltransferase, which resulted in increased trisaccharides and slightly higher sucrose levels (Nicholson, 2007). Other similar transgenic studies, which were rather unsuccessful in increasing sucrose levels in sugarcane include the reduction of soluble acid invertase activity, (Botha *et al.*, 2001) as well as the over expression of SPS (sucrose phosphate synthase) (Grof *et al.*, 1998).   One recently published patent (Boussiengui-Boussiengue and Kossmann, 2014) claimed that silencing a uridine monophosphate synthase (UMPS) gene led to significantly increased sucrose content.   Another approach modified sugarcane by introducing a bacterial sucrose isomerase enzyme into the plant's system, which then produced isomaltulose (IM) from sucrose (Lina *et al.*, 2002). Isomaltulose (IM) cannot be metabolised or transported in plants, but its exogenous application triggers some plant sugar-sensing mechanisms and changes gene expression profiles (Loreti *et al.*, 2000; Sinha *et al.*, 2002). IM is digested more slowly by the intestinal sucrase/isomaltase, resulting in less fluctuation in blood sugar levels and insulin concentrations (Lina *et al*, 2002). A study conducted by Wu and Birch (2007) introduced the sucrose isomerase gene in sugarcane. In this study, IM was accumulated in storage tissues without any decrease in stored sucrose concentration, therefore doubling the total sugar concentration in harvested juice.

Induced mutagenesis (random induced genome mutations), followed by selection for valuable characteristics have been exceptionally successful in producing plants with useful traits (Haughn and Somerville, 1986; Irvine *et al.*, 1991; Zambrano *et al.*, 1999; Zambrano *et al.*, 2003; Ali *et al.*, 2007; Punyandee *et al.*, 2007; Kenganal *et al.*, 2008; Rai *et al.*, 2011). Mutation breeding is an alternative solution for creating non-transgenic crops with desired traits not subjected to transgenic crop regulations and restrictions. Mutations can either be induced physically or chemically. Physical mutagenesis (irradiation) can be administered either with non-ionising agents (ultra-violet (UV) light), ionising agents (X and gamma rays, alpha and beta rays) or with high linear energy transfer (LET). Irradiation usually results in large-scale deletions, high chromosome aberration frequencies and high levels of non-repairable DNA damage in plant genomes (Parry *et al.*, 2009).   Whereas chemical mutagenesis, specifically in plants, is largely administered using mutagens such as, ethylmethane sulfonate (EMS), methylmethane sulfonate (MMS), hydrogen fluoride (HF), sodium azide, N-methyl-N-nitrosourea (MNU) and hydroxylamine. The application of these agents results mainly in single nucleotide DNA base pair mutations. Chemically induced mutations are generally of more use because, unlike physically induced mutations, they are

easier to administer, do not require specialised expensive equipment and result in high mutagenicity rates at random sites, with low mortality rates (Koch *et al.*, 2012).

Mutation breeding strategies have been applied in the past only in a limited manner to sugarcane and can potentially be exploited to a greater extent in this crop species. For example, a review of the FAO/International Atomic Energy Agency's Mutant Varieties Database showed only 13 induced sugarcane mutant accessions among more than 3000 registered mutants from over 170 different plant species (Rutherford *et al.*, 2014). These approved sugarcane mutant lines include traits ranging from high sucrose and cane yield to pest and disease resistance (FAO/International Atomic Energy Agency (IAEA) mutation variety database). Additional research examples of mutagenesis applied in sugarcane include the use of EMS to produce sugarcane tolerant to herbicides such as imazapyr (Koch *et al.*, 2012), plants resistant to sugarcane mosaic virus (SCMV) (Zambrano *et al.*, 2003), selection and characterisation of sugarcane mutants with improved resistance to brown rust (Oloriz *et al.,* 2012) as well as application of radiation to induce mutations *in vitro* in sugarcane (Patade and Suprasanna, 2008).

1.1.4 Addressing two specific issues of relevance to the sugarcane industry:

1) Sugarcane's viability as a feedstock for biofuel production

Current environmental degradation due to factors such as CO_2 emissions (global warming), drought, the growth of the human population with the accompanying increase of food supply demands as well as fossil fuel implications, all lead to an urgent call for better, alternative sources of energy. Fossil fuels have been used worldwide as the main source of energy for centuries mainly because they produce large amounts of concentrated energy at a relatively low cost. However, fossil fuels have negative environmental implications causing air, water and land pollution, they also contribute largely to the CO_2 emissions that have added to global warming and climate change, all of which in turn compromise human health (http://biofuel.org.uk/). Therefore, the rapidly growing population as well as the simultaneous need to protect the environment, rather render the use of fossil fuel as an energy source less popular.

These implications have therefore led to the search and exploration for alternative sources of energy to replace fossil fuels. An alternative source of energy should be renewable, in constant supply, environmentally friendly, should not impede on food production and be produced and consumed at relatively low costs (http://biofuel.org.uk/). The production of biofuels has been the most attractive alternative source of energy considering the afore-

mentioned requirements although their advancement has been controversial due to food supply demand as well as the fact that biofuels are primarily produced from food sources such as maize and sugarcane (Naik *et al.*, 2010; http://biofuel.org.uk/).

According to The Statistics Portal 2014 report, countries leading in biofuel production (http://www.statista.com/statistics/274168/biofuel-production-in-leading-countries-in-oil-equivalent/) included the United States of America (USA), Brazil and some European countries such as Germany, France and the Netherlands. The USA led with production of approximately 30 billion tons of biofuel, about 42.5% of the total globally produced biofuel. In South Africa, over the past few years government sought to introduce policies regarding the national biofuel industry by first drafting the *White Paper on Renewable Energy* (2003) and more recently the *Position Paper on the SA Biofuels Regulatory Framework* (2014) (http://www.gov.za/sites/www.gov.za/files/37232gen24.pdf), which introduced biofuel mandatory blending with petrol and diesel, set to take effect in October 2015. The production of biofuel is supposed to account for 2% of existing fuel volumes grown on 1.4% of total arable land, with crops including sorghum, sugarcane and sugar beet being used to produce bioethanol, and canola, sunflower and soya beans for biodiesel production. Maize, a globally common first-generation biofuel production crop is excluded from the list owing to its pivotal role as one of the country's most important food sources. Its use would detrimentally affect the food security, while other potential source plants such as the Jatropha, which produces seeds rich in oil, are excluded for biodiversity and environmental concerns. The future aim of this strategy is to produce approximately 400 million litres of biofuels per annum on 14% of arable land, which would in turn assist alleviate unemployment in South Africa (Burger, 2014).

There are three generations of biofuels that have been developed or investigated so far. First- and second-generation biofuels use food supplies and by-products of crops, used as food supplies, respectively as source material, both are implemented and already in use in countries such as USA and Brazil. Third-generation biofuels are, however new to the biofuel mainstream and are not yet commercialised. These are made from algae and show advantages over the first two types in terms of producing very high biofuel yields with lower resource inputs. With this method a diverse array of fuels can be produced including ethanol, diesel, butanol, gasoline, jet fuel, methane and vegetable oil, without competing for arable land. However, they have one disadvantage that limits their implementation, namely that algae require large amounts of water, nitrogen, and phosphorus to grow. The amounts of fertiliser needed would produce more greenhouse gas emissions than would be saved with algae-based biofuels. This also means that the cost of producing algae-based biofuel is

much higher than that of the other feedstocks (http://biofuel.org.uk/; Mata *et al.*, 2010; Dragone *et al.*, 2010).

First-generation biofuels use food sources such as sucrose from sugarcane and/or sugar beet, oil from soy beans or sunflowers and starch from maize to produce energy. The use of food sources, together with the fact that the general process of growing and processing crops for biofuel production is expensive (Naik *et al.*, 2010; http://biofuel.org.uk/), raised questions regarding the production of this generation of biofuels as alternative energy source. However, countries such as USA and Brazil, currently still produce bioenergy from food sources, especially maize (corn) and sugarcane (Mejean and Hope, 2010). Nevertheless, different means of sourcing energy that do not compete with food supplies, yet are still cost effective are vital. This demand led to the development of second-generation biofuels (Naik *et al.*, 2010; http://biofuel.org.uk/).

Production of bioenegy is based on fermenting sugars to ethanol and the source of fermentable sugars (starch or sucrose) determines the energy balance of the source crop (Naik *et al.*, 2010; Zabed *et al.*, 2014). Energy balance refers to the difference between the energy required to convert the source crop into bioenergy and the amount of energy released from the crop. For example, sugarcane-based bioenergy has seven times more energy balance compared to that of maize, primarily because starch first has to be converted to sugars before it can be distilled to ethanol (Mejean and Hope, 2010). This significantly increases maize-based bioenergy production costs as opposed to sugarcane-based ones, as the latter produces sugars which can be directly distilled into ethanol. Additionally, sugarcane-based bioenergy is more productive as, on average, a hectare of sugarcane produces twice as much bioenergy as its maize counterpart (Mejean and Hope, 2010).

Second-generation biofuels use plant lignocellulosic components, the by-products of plants used as food sources or non-food plants, as source material (Naik *et al.*, 2010). As such, sugarcane has been found to be a suitable candidate to produce second-generation biofuel (Waclawovsky *et al.*, 2010). This crop is generally considered as high yielding, rapidly growing and it requires relatively small energy and labour inputs during its growth and harvest (Naik *et al.*, 2010; Waclawovsky *et al.*, 2010). However, although bagasse (soft-wooded sugarcane waste material recovered after sucrose extraction for food sources) shows positive attributes, it possesses a cell wall of which the biological structure is difficult to break down, making the process of extracting lignocellulosic components expensive. This is specifically due to the recalcitrance of lignin, a very important component that provides the structural and mechanical support of the cell wall and assists in transportation of water and

nutrients throughout the plant (Chabannes *et al.*, 2001; Jones *et al.*, 2001; Sarkanen and Ludwig 1971).

The process of fermenting lignocellulosic biomass, obtained from a milled biofuel source crop such as sugarcane baggase, to ethanol requires three steps: pre-treatment of biomass, acid or enzymatic hydrolysis and fermentation (Naik *et al.*, 2010). Of these steps, pre-treatment is the biggest rate limiting step as it breaks down the lignin structure and disrupts the crystalline cellulose microfibers to allow acids and enzymes to easily access and hydrolyse cellulose as shown in Figure 1.1 (Kumar *et al.*, 2009; Naik *et al.*, 2010; Mosier *et al.*, 2005). Several types of pre-treatments have been developed of which steam explosion is most commonly used (Kumar *et al.*, 2009; Naik *et al.*, 2010). In steam explosion, biomass is placed in a pressure vessel and is vaporised using saturated steam for hemicellulosic hydration. The process is terminated by a sudden drastic pressure drop, exposing the material to normal atmospheric pressure, therefore causing a heat explosion that degrades hemicellulose, transforms lignin's structure and, thus, increases the potential of cellulose hydrolysis (Figure 1.1). Hemicellulose is thought to be hydrolysed by acetic acid and other acids released during steam explosion while hydrolysis of hemicellulose and its removal from the microfibrils exposes cellulose to enzymes. Lignin is partially removed during pre-treatment but is redistributed on the fibre surfaces as a result of melting and subsequent depolymerisation/repolymerisation reactions (Naik *et al.*, 2010; Kumar *et al.*, 2009). Sugars (xylose) produced at this stage can be fermented to ethanol (Figure 1.2) or the crystalline cellulose, which still remains solid after pre-treatment, can be broken down to glucose by enzymatic hydrolysis which is then in turn fermented to ethanol.
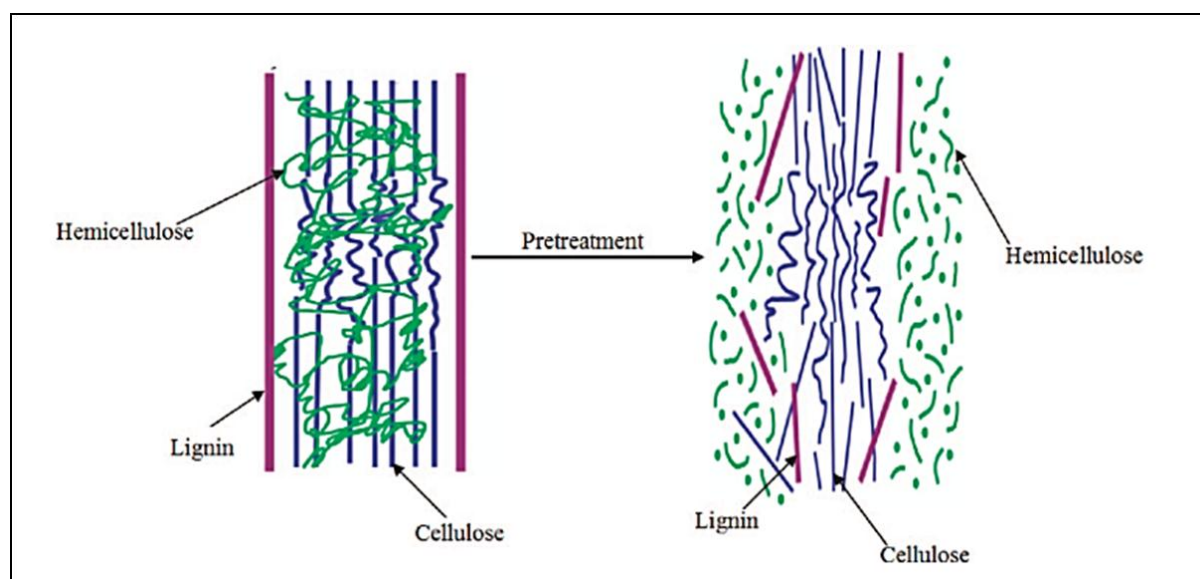
Figure 1.1: A schematic representation of the role pre-treatment plays in biofuel conversion from biomass (Hsu *et al.*, 1980).
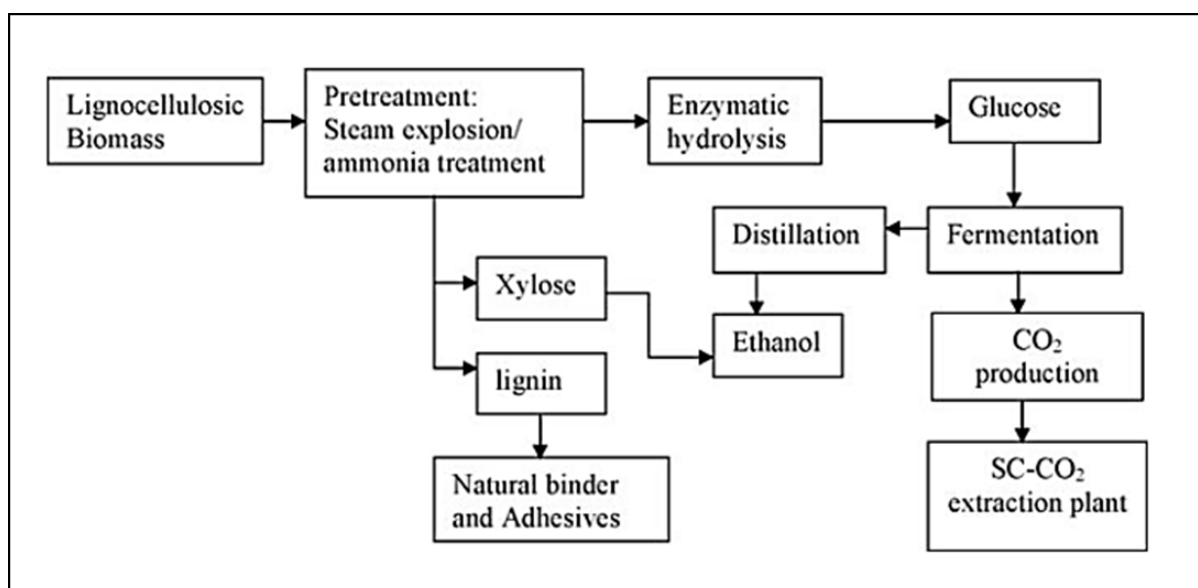
Figure 1.2: A schematic representation of bioethanol production from lignocellulosic biomass (Naik *et al.*, 2010).

Through genetic engineering a few studies have been conducted that aim to overcome lignin recalcitrance and improve extraction of lignocellulosic biomass by modifying the expression of genes or enzymes vital to cell wall biosynthesis. For example, Chen *et al.* (2006) down-regulated seven enzymes of the lignin biosynthesis monolignol pathway using antisense and/or RNA interference (RNAi) in alfalfa (*Medicago sativa*). The plants were later analysed by Chen and Dixon (2007) to determine the relationship between lignin content, chemical/enzymatic saccharification and crop yield in mature alfalfa stems. They determined that down regulating enzymes such as caffeic acid 3-*O*-methyltransferase (COMT) and caffeoyl CoA 3-*O*-methyltransferase (CCoAOMT) as well as hydroxycinnamoyl CoA: shikimate hydroxycinnamoyltransferase (HCT) results in enzymatic saccharification efficiencies almost double that of the control while HCT down-regulation also results in decreased total lignin. In addition to these observations, plant yield was reported not to be affected and plants demonstrated increased fermentable glucose production. Another study also successfully used RNAi to reduce lignin recalcitrance in sugarcane (Jung *et al.*, 2012). This demonstrated that 67 – 97% down-regulation of sugarcane COMT reduced lignin content by 3.9 – 13.7%. The syringyl/guaiacyl ratio in lignin was reduced from 1.47 to 1.29 and 0.79. Furthermore, the yield of directly fermentable glucose increased up to 29% and 34% with or without dilute acid pre-treatment respectively. These studies show that genetic manipulation of the monolignol biosynthesis pathway can reduce lignin recalcitrance in lignocellulosic biomass without compromising plant performance (Hoang *et al.*, 2015; Jung

et al., 2012)*.* Sattler *et al.* (2014) isolated EMS mutagenised putative sorghum *brown midrib* (*bmr*) mutants and characterised them based on their leaf midrib phenotype and allelism tests using previously described sorghum *bmr* mutants *bmr 2*, *bmr 6* and *bmr 12* (COMT gene). Out of 46 putative mutant lines, four *bmr* lines showed reduced lignin concentration when compared to the control. Like the reference mutants, the four selected mutants may affect monolignol biosynthesis and may be useful for bioenergy and forage improvement.

Apart from lignocellulosic biomass, sucrose is also fermented to ethanol, which can be converted to biofuels. In countries that still produce first-generation biofuels such as USA and Brazil, sugarcane breeders have focused on increasing sucrose yield to increase ethanol production for biofuels (http://isaaa.org/resources/publications/pocketk/45/default.asp). However, less progress has been made in this regard because of concerns of food security (Smith, 2008). Studies that focus on increasing sucrose yield in sugarcane aim to support food security rather than biofuel production. However, one study aimed to investigate the effects of manipulation of plant cell wall synthesis, particularly the uridine 5'-diphosphate (UDP)-glucose (Glc) through down-regulation of UDP-glucose dehydrogenase (UGD) in sugarcane. This study hypothesised that a decrease in carbon flux through repressing UGD would increase UDP-glucose, the substrate for sucrose synthesis and subsequently the accumulation of sucrose (Bekker, 2007). The study showed a correlation between increased sucrose and cellulose accumulation and decreased UGD activity in transgenic lines. In addition, an increase in uronic acids such as galacturonic and glucuronic acids were also observed in transgenic lines. To determine whether the increase in uronic acids could be attributed to upregulation of the *myo*-inositol oxidation pathway (MIOP), the *myo*-inositol oxygenase (MIOX) expression and activity were determined. The results showed that both the expression and activity of the MIOX were upregulated in the transgenic lines. This observation led to a hypothesis that when the sugar nucleotide oxidation pathway (SNOP) is repressed due to down-regulation of UGD, the MIOP compensates for the synthesis of UDP-glucuronic acid (Glc A), a precursor for cell wall sugars and lignocellulosic biopolymers. This pilot study therefore needs further investigation to unravel the details correlating the sugar nucleotide interconversion pathways (SNOP and MIOP) as well as sucrose accumulation with cell wall biosynthesis to assist in biofuel production.

2) Sugarcane and drought environmental factors

Decreased water availability due to reported changes in climate has resulted in drastic variations in precipitation levels which have directly influenced the anthropogenic release of CO_2 (Ryan, 2011). Correspondingly, plant drought stress is a serious agronomic problem

contributing to severe loss in plant yield and productivity (Fisher and Turner, 1978; Le Rudulier *et al.*, 1984; Delauney and Verma, 1990). Agricultural drought and water deficit as described by Levitt (1972) states that when water in the plants environment becomes deficient, plant transpiration cannot fully meet atmospheric demand, and a plant water deficit evolves. Water deficit put a strain on the plant that causes damage and propels a network of gene responses proportional to the severity of the deficit. The plant can cope with the stress either by avoiding or tolerating the strain. Drought tolerance therefore, consists of two components: dehydration tolerance, which is the plant's capacity to reduce or avoid the water deficit. The other component is dehydration resistance, which is the plant's capacity to sustain plant functions under water deficit.

In South Africa, of the land available for commercial sugarcane cultivation, approximately 85% is rain-fed regions that include the coastal KwaZulu Natal region, where 80% of the sugarcane is grown and the midlands regions (South African Sugarcane Association (SASA) 2010/2011). The 15% of irrigated land for sugarcane cultivation is mostly located in Mpumalanga and Pongola, the north-eastern regions of South Africa (SASA 2010/2011). Crop production in the rain-fed regions is especially undermined by inconsistent precipitation levels. In the last three decades one severe drought was recorded in each decade for the southern coastal areas of South Africa, the recovery from each of these has taken a number of years and cane production in these regions were negatively influenced (SASA 2010/2011). A recent report from Alberts and Mokhema (2015) mentioned that drought experienced in South Africa has cut the production of sugarcane in KwaZulu Natal by 34%. As opposed to 21 million tons of sugarcane produced in 2012/2013, only 17.76 million tons have been produced in the years 2014/2015. Furthermore, Gosal *et al.* (2009) and Morison *et al.* (2008) estimated production and yield losses can be as high as 70% due to water deficit during sugarcane development. It is therefore necessary to acknowledge that drought stress is a serious agronomic problem which greatly influences the crop growth, yield and production.

Sugarcane development can be categorised into four stages (Figure 1.3), all of which are influenced to different extents by abiotic stress, namely:

1) The germination and establishment phase: Under field conditions, this process starts seven to ten days after planting the setts and usually lasts for 30-35 days, which is usually after completion of bud germination (Smit, 2010; Pierre *et al.*, 2014). Germination consists of activation and subsequent sprouting of vegetative buds. This process is influenced by external factors such as aeration, soil temperature and moisture as well as internal factors

such as bud health, sett nutrient status, sett reducing sugar content and sett moisture. Temperatures 28-30°C as well as humid soils ensures rapid germination.

2) The tillering phase: A physiological process of repeated underground branching from compact nodal joints of the primary shoot that starts from around 40 days after planting until day 120. Its purpose is to provide the crop with an appropriate number of stalks required for good yields. Factors such as variety, light, temperature, water availability and fertiliser greatly influence this process. Adequate light reaching the base of the sugarcane is of vital importance in tillering, hence of all the external factors, light is the most important. Temperatures below 20°C result in retardation of stalk growth, while temperatures around 30°C result in optimal growth of stalks. Usually, a maximum tiller population is reached at 90 – 120 days after planting and at about 150 - 180 days, at least half of the tillers die to establish a stable population. This population is cane that can be milled. Practices such as spacing, time of fertigation, water availability and weed control also influence tillering.

3) The grand growth phase: The most important phase of crop growth, where actual cane growth and elongation occurs meaning that yield build-up takes place. This phase starts at 120 days (tillering) after planting and usually lasts up to 270 days in a 12 month sugarcane growth cycle. Leaf production is frequent and rapid during this phase. Conditions such as drip irrigation, fertigation and warm, humid and sunny climate favours favour cane elongation. Temperature of 30°C with 80% humidity is conducive for good growth. It is apparent that moisture (water) stress reduces elongation of internodes and therefore yields.

4) Ripening and maturity phase: In a 12 month crop cycle, this process starts ~270 days after planting and takes about three months. Here, sugar synthesis and accumulation takes place, with reduced vegetative growth. As ripening advances, glucose and fructose are converted to sucrose. Ripening proceeds from the bottom to the top internodes, therefore the mature internodes contain more sucrose than intermediate and young internodes. Sunlight and dry weathers are highly conducive for ripening, which means this stage is the least sensitive towards water limiting conditions. The vast amount of growth, development, and sugar accumulation occurs during the tillering and grand growth stages. These stages, as afore-mentioned are dependent on water availability to complete development and are therefore the most drought-sensitive. It is consequently clear that water deficit, either temporary or permanent, can negatively affect sugarcane yield and product quality (Ramesh, 2000).
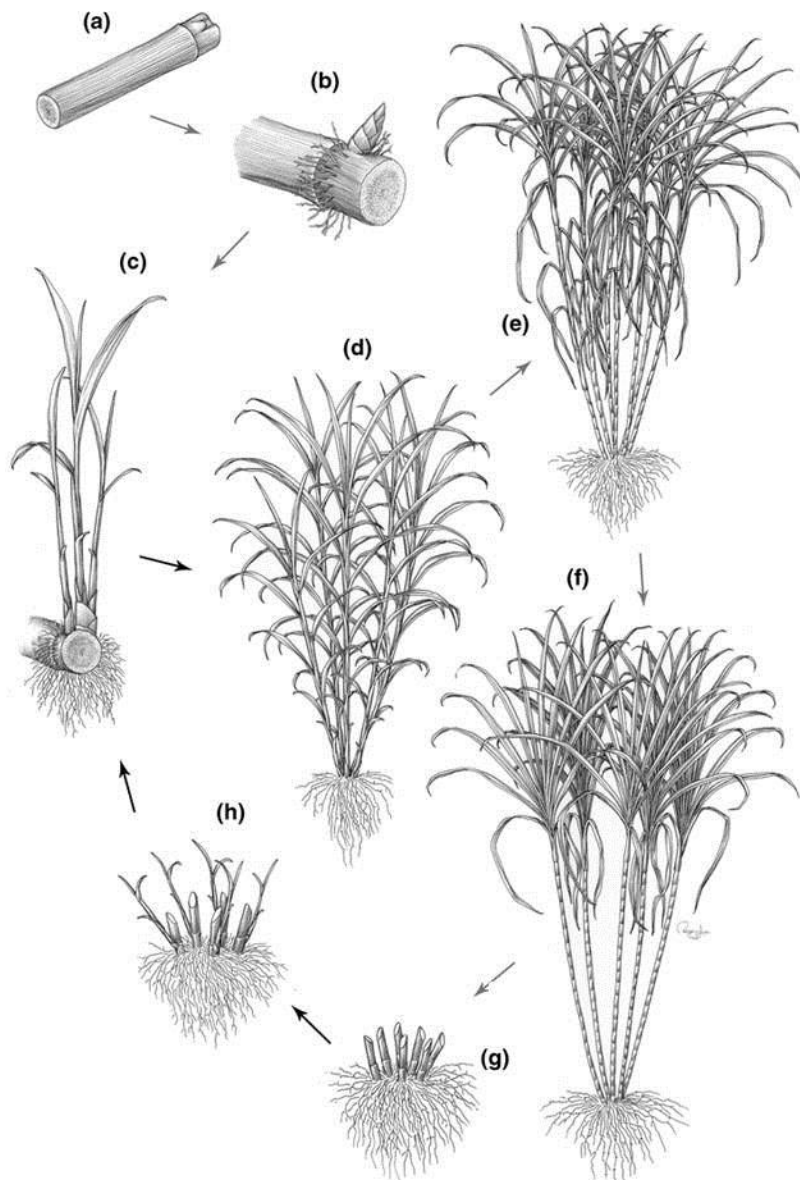
Figure 1.3: A representation of sugarcane growth stages during 12 months of development. a) Stalk pieces used for planting; b) beginning of bud sprouting and rooting; c) tillering initiation; d) intense tillering; e) grand growth; f) maturation; g) harvest; h) ratoon sprouting (Cheavegatti-Gianotto *et al.*, 2011; http://www.sugarcanecrops.com/crop_growth_phases/ripening_maturation_phase/).

Decreasing sugarcane productivity propelled by the lack of water has led to the undertaking of research to seek alternative measures to produce sugarcane cultivars tolerant to drought conditions. To date, there have been reviews published outlining the possibilities for inducing abiotic stress tolerance in plants using genetic engineering, but the actual production of plants with demonstrably improved abiotic stress tolerance has been slow (Blum, 2014). This delay is in part, due to the frequently observed growth and/or yield penalty in the crop plants when grown in conditions of non-stress as well as the complexity of manipulating

cascades of genes involved with potential to improve tolerance during transgenic alterations (Tester and Bacic, 2005).   In sugarcane for example, Kumar *et al.* (2014) genetically transformed sugarcane with the *Arabidopsis* vacuolar pyrophosphatase (AVP1) gene, where transgenic lines showed a longer and profuse root system when compared to the wildtype. These transgenic lines were able to withstand salt and water stress. Zhang *et al.* (2008) used the *Grifola frondosa* trehalose synthase gene in attempts to improve drought tolerance. In this regard, transgenic plants accumulated high levels of trehalose, which resulted in increased drought tolerance when compared to non-transgenic plants that did not show detectable trehalose. To date, only one genetically modified sugarcane cultivar reported to have increased drought resistance has been released for commercial use (Noguera, *et al.*, 2015). This variety is characterised by expressing the *choline dehydrogenase (betA)* gene, which leads to the accumulation of membrane-protectant glycine betaine, therefore conferring drought tolerance. However, this trait does not mean the plants can be grown with less water, or use water more efficiently. It only provides the cane with some modest protection against modest drought conditions. Traditional breeding techniques have been much more successful in maintaining yields during dry periods (Richardson, 2014). However, transgenic crops still have the potential to counter abiotic stress tolerance.

An alternative method to transgenics is mutagenic treatments followed by the selection for improved abiotic stress tolerances in mutant plants. In the past mutation breeding has also been used to develop a number of crop cultivars tolerant to drought stress conditions (Oo *et al.*, 2015; Njau *et al.*, 2005). Some related studies in sugarcane have been executed, for example, Nikam *et al.* (2015) and Patade *et al.* (2008) introduced salt tolerance in sugarcane using gamma radiation. As opposed to genetic modification, mutagenesis is not restricted by government regulations in terms of product release for commercial purposes. Although studies have been conducted to increase abiotic stress tolerance in crops, more effort in this regard is still required.

1.2 Project Rationale and Objectives

With the increasing commercial demand for sugarcane, traditional methods alone do not have the capability to meet the required need for this crop. The use of biotechnological technologies can complement traditional breeding methods. This project therefore aims to apply biotechnological methodologies for sugarcane enhancement. The project is divided in two parts, the first part aims to produce drought tolerant sugarcane by means of mutation induction, while the second part aims to increase the viability of sugarcane plants as second-generation biofuel sources using genetic engineering. This project was executed on

Saccharum hybrid species using the standard NCo310 and South Africa originating N19, N40 and NCo376 cultivars.

Part 1

Using mutation breeding to develop drought tolerance in sugarcane

This part of the project was divided into five portions to assist in selecting potentially drought tolerant sugarcane mutant lines originating from the different *Saccharum* hybrid cultivars, NCo310, NCo376, N40 and N19, respectively.

The following objectives were included:

- Establish optimum *in vitro* growth parameters, for cultivars N19, N40 and NCo376 using different 2,4-D concentrations incorporated in the culture media for callus induction and subsequent plantlet regeneration.
- Expose callus from sugarcane cultivar NCo310 to a range of EMS concentrations, to determine the optimum EMS concentration for mutation induction required for *in vitro* osmotic tolerance.
- Expose NCo310 callus to PEG (polyethylene glycol) and mannitol at different concentrations, to determine the optimum concentration to select osmotic tolerant clones *in vitro*.
- Expose sugarcane callus from sugarcane cultivars NCo310, NCo376 and N19, to pre-determined EMS and osmoticum concentrations, to select osmotic tolerant clones *in vitro*.
- Lastly, EMS treated plantlets that survive the *in vitro* osmotic selection were exposed to *ex vitro* pot drought trials to confirm drought tolerance in selected plantlets.

Part 2

Overexpressing a glucoronokinase gene from Arabidopsis in sugarcane

Bekker (2007) conducted a study which demonstrated that, upon repression of the uridine 5'-diphosphate glucose dehydrogenase (UGD) gene, which form part of the sugar nucleotide oxidation biochemical pathway (SNOP), the following genes were upregulated: genes involved in sucrose synthesis namely, sucrose synthase (SuSy) and sucrose phosphate synthase (SPS); and *myo*-inositol oxygenase the first enzyme involved in the *myo*-Inositol

oxidation pathway (MIOP). This observation then led to the hypothesis that when the SNOP is repressed, the MIOP possibly assumes the function of producing sucrose in sugarcane. Therefore, this part of the project aimed to further investigate the role of glucuronokinase, the second enzyme involved in the MIOP and its possible effect on sucrose content in sugarcane plants. Glucoronokinase plays a role in producing cell wall sugars and lignocellulosic polymers. For this, a glucoronokinase gene from *Arabidopsis thaliana* was over expressed in sugarcane and initial sucrose content measurements taken.

The following objectives were included:

- Transform sugarcane with the glucuronokinase gene isolated from *Arabidopsis thaliana* using particle bombardment.
- Perform molecular analysis to confirm transformation and expression levels of the transgene in the transgenic sugarcane lines.
- Determine sucrose content, enzymatic activity and phenotypic variations in the transgenic sugarcane.

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Chapter 2

Manipulation of sugarcane to improve drought tolerance by mutagenesis

2.1 Introduction

Worldwide crop production is restricted by limited water resources. Competition for water, irrigation limitations, global temperature increases and inconsistent precipitation levels all contribute to the challenge of producing crops with inadequate water resources (Vadez *et al.*, 2014).   As previously mentioned, sugarcane hybrids used primarily for commercial purposes are optimally cultivated in tropical or sub-tropical regions due to their water requirements. They are therefore sensitive to drought stress and losses due to this are not unusual, occurring almost annually in Southern Africa. Sugarcane farmers also do not have the luxury of avoiding annual drought seasons due to cane maturation stretching across 12 to 18 months (Gentile *et al.*, 2015).

Among the methods used to improve sugarcane traits is mutation breeding, also known as induced mutagenesis, a process where genetic material of an organism is altered either physically or chemically. Physical mutation breeding can be performed with irradiation using non-ionising agents such as ultra-violet (UV) light and ionising radiation agents such as X and gamma rays. On the other hand chemical mutation breeding in plants, normally uses mutagens such as ethylmethane sulfonate (EMS), methylmethane sulfonate (MMS) or hydrogen fluoride (HF). Among the chemicals, EMS is the preferred mutagen used in studies because it generates random small-scale or point mutations at high frequencies (Schy and Plewa, 1989) alongside low chromosomal aberration frequencies (Van Harten 1998), which are mostly non-lethal at intermediate concentrations. EMS has been used in the past to introduced useful traits in a number of plant species. For example, in banana (*Musa* spp.) it was used to induce genomic variability resulting in drought tolerance *in vitro* (Badibadi *et al.*, 2012), rice (*Oriza sativa*), where EMS was used on mature seed-derived calli for rapidly obtaining TILLING (targeting induced local lesions in genomes) mutant populations (Serrat *et al.*, 2014) and maize (*Zea mays*) where EMS sensitivity was determined as a factor contributing to albino leaf stripes (Efron, 1974). EMS is most effective when applied to dividing cells as this is the stage where cells are engaged in DNA replication and the probability of incorrectly repairing a mutation is highest (Kilbey and Hunter, 1983). In sugarcane, to this point, examples of mutagenesis in sugarcane include

the use of EMS to produce sugarcane tolerant to herbicides such as imazapyr conducted by Koch *et al.* (2012). EMS has also been successfully used to produce plants tolerant to a range of various stresses such as salt stress (Kenganal *et al.*, 2008).

In order for mutation breeding to be successful, a high through put and efficient selection system must be in place to identify potential genetic clones displaying desirable phenotypes. To simulate or mimic a drought stress environment *in vitro*, an appropriate osmoticum (a substance that modifies osmotic potential of nutrient solution in plant cultures) should be an inert, non-ionic, long chain polymer that dissolves in water and modifies osmotic potential of a nutrient solution in a relatively controlled manner. Furthermore, the osmoticum should not be taken up by the cells as it might induce cellular toxicity. Among most used osmotica, only PEG meets all the requirements (Tsago *et al.*, 2013; Rao and Jabeen, 2013; Errabii *et al.*, 2007; Lawlor, 1970). PEG is available in a range of molecular weights (4000 – 8000 g/mol), however, for drought experiments, a molecular weight higher than 4000 is required to induce water deficit in plants by decreasing the water potential of nutrient solutions (Blum, 2004), without the cultured cells taking it up which can lead to cellular toxicity (Lawlor, 1970). Some osmotica, such as glucose, sucrose and mannitol, although used in numerous plant cell culture studies as compatible solutes, have been proven to be taken up by the plant and, therefore may, at certain concentrations, induce cell toxicity (Hassan *et al.*, 2004; Blum, 2004). Therefore, these chemicals are not always ideal to use for *in vitro* selection as they may produce false positive results, especially in stress-related studies.

To select for drought tolerance using mutagenized tissue *in vitro*, the optimum concentration of the mutagen should ideally produce genomic mutations at a high rate without compromising the survival of the tissue. The optimum concentration of the osmoticum on the other hand, should ideally, be high enough to result in the death of wild type tissue after a certain time point. This combination would then ensure that the survival of any EMS treated tissue selected on the osmoticum, will likely be due to the mutations induced in the tissue's genomic material, rather than insufficient osmoticum concentrations and/or exposure time periods.

Studies that describe *in vitro* osmotic selection using chemical mutagenesis in sugarcane have not yet been reported. Some related studies, have, however been executed, for example, Bidabadi *et al.* (2012) selected and characterised water stress tolerant lines among EMS induced banana variants *in vitro*. For this, shoot apical meristems from the banana cultivars, 'Berangan Intan' and 'Berangan', were used as explants and exposed to EMS concentrations ranging from 150 to 250 mM for 30 to 60 mins. After *in vitro* mutagenesis and multiplication, banana variants were then exposed to PEG at concentrations ranging

between 10 and 30 g/L and cultured for one month. Resulting mutant plantlets showed a significant decrease in leaf water content compared to susceptible and non-mutated lines. In sugarcane specifically, Nikam *et al.* (2015) introduced an *in vitro* mutagenesis system for salt tolerance and other agronomic characters using radiation (gamma rays). Rao and Jabeen (2013) developed a system for *in vitro* selection of drought-tolerant sugarcane callus clones among somaclonal variants using 20-30% (w/v) PEG (6000) as the selective agent. The results of that study showed that selected callus lines grew significantly better than non-selected (control) callus on PEG but regenerated plants were never submitted to an *ex vitro* drought pot trial to confirm drought tolerance.

This study, by means of chemical mutagenesis, aims to produce drought tolerant sugarcane plants using *in vitro* osmotic selection followed by plant regeneration and preliminary glasshouse drought pot trials. To achieve this we firstly focused on optimising and determining the *in vitro* parameters for sugarcane cultivars N19, NCo376 and N40 to enable us to grow callus and effectively mutant and select for *in vitro* stress tolerance. According to the South African Sugarcane Research Institute (SASRI), the included cultivars have different field drought sensitivity levels but, there *in vitro* growth parameters have not yet been established.

Secondly, we focused on exposing callus from the model sugarcane cultivar NCo310 to different EMS concentrations to determine the optimum mutagen concentration. The ideal EMS concentration should induce high mutation rates in the sugarcane genome without compromising in vitro cell survival

Thirdly we focused on *in vitro* kill curves, using the NCo310 sugarcane cultivar as a model, for two different osmotica, namely mannitol and polyethylene glycol (PEG). Different concentrations of these osmotica were tested in order to establish both the best suited osmoticum and optimal concentration to be used on sugarcane callus to identify potentially drought tolerant clones *in vitro.*

The established *in vitro* growth parameters, optimal EMS concentration and ideal osmoticum were then used to mutate sugarcane callus (NCo310, NCo376, N40 and N19) and identify plantlets with enhanced in vitro drought tolerance. These surviving plantlets were hardening off in the greenhouse and preliminary drought pot trials were performed to corroborate enhance drought tolerant phenotypes.

2.2 Materials and Methods

2.2.1. Plant Material

Saccharum species hybrid cultivars NCo310, NCo376, N19, N40, with different levels of drought tolerance, were received from the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, KwaZulu Natal, South Africa. These included the drought susceptible N19 and N40 cultivars and the highly drought susceptible NCo376 cultivar. The NCo310 cultivar was used as model cultivar for all *in vitro* osmotic selection trials and is considered as relatively drought tolerant.

2.2.2. *In vitro* cultivation of sugarcane

2.2.2.1 Explant preparation and callus induction

For *in vitro* callus induction, immature inner leafs from the apical segment of sugarcane stalks were used as explant material. The basal part of the inner leaf roll was aseptically isolated from the explant, sliced into approximately 3 – 5 mm thick leaf roll discs and placed on MS media (pH 6.0) containing 4.4 g/L MS with vitamins (Murashige and Skoog, 1962), 20 g/L sucrose, 0.5 g/L casein, different 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations (explained below), and 2.2 g/L gelrite. The media was autoclaved for 15 min at $121^{o}C$. All chemicals and hormones were purchased from Sigma Aldrich (St. Louis, USA) unless otherwise stated.

To determine the optimal auxin concentration for callus induction for each drought susceptible sugarcane cultivar, leaf disks from the three cultivars were placed on five different 2,4-D concentrations, namely 0.5, 1, 2, 3 or 4 mg/L ($MS_{0.5}$, MS_1, MS_2, MS_3 and MS_4). Leaf discs were kept in the dark at $26^{o}C$ for six to eight weeks and sub-cultured fortnightly for the duration of the callus induction phase. After approximately five to six weeks of induction, as callus began to develop from the leaf roll discs, callus was removed from the leaf roll discs using a scalpel and tweezers and placed directly onto the media for additional growth and multiplication. Leaf disk lethality and callus formation were assessed after eight weeks for each cultivar and 2,4-D concentration used.

2.2.2.2 Regeneration of in vitro plantlets

After eight weeks of induction in the dark, callus that proliferated from discs were aseptically transferred to basic MS media without 2,4-D and placed under cool white fluorescent light (Osram; 36W/640) with intensity of 50 μmol photons/m^2/sec at $26^{o}C$ with a 16/8 hour

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light/dark regime, for 14 weeks of plantlet formation and development. Callus was covered with thin yellow cellophane filter layer to lower the light intensity and to decrease light stress. Cultures were sub-cultured every second week.

After 14 weeks of regeneration the efficiency of *in vitro* somatic embryogenesis and plant regeneration for each cultivar across all 2,4-D concentrations was evaluated.

2.2.2.3 Analysis of In vitro growth efficiency

The *in vitro* efficiency coefficient for each cultivar was calculated, based on a formula adapted from Miroshnichenko *et al.* (2013). For each respective cultivar and each 2,4-D concentration tested, the callus induction frequency (C), per 30 leaf discs explants, indicating the number of leaf discs that produced callus; the embryogenicity frequency (E) providing an indication of embryogenic callus produced; and the plantlet formation frequency (S) per embryogenic calli accounting for the number of green shoots produced from the original 30 leaf discs, were calculated. Furthermore, the resulting differences in shoot length (L) and the number of genetic off-type shoots (W) observed per plate for each 2,4-D concentration per cultivar were also documented. The *in vitro* efficiency coefficient for each cultivar was calculated using the formula (C/10)*(E/10)*(S/10)*L (cm) (Miroshnichenko *et al.*, 2013).

2.2.3. *In vitro* NCo310 exposure to ethyl methane sulfonate (EMS)

The NCo310 sugarcane cultivar was used as a model cultivar to determine the ideal concentration of EMS for *in vitro* mutagenesis of sugarcane callus. The apical meristem was acquired and callus induced as previously described in section *2.2.2.1*. To determine the optimal EMS concentration for *in vitro* mutagenesis, embryogenic callus was harvested and exposed to EMS based on the method described by Koch *et al.* (2012). EMS concentrations investigated were 5, 10, 20, 25, and 50 mM.

Liquid MS_3 media (basic MS media with 3 mg/L 2,4-D) was prepared as described above. After autoclaving, 10 ml of the media was aliquoted into 50 ml tubes, and EMS was added to the liquid MS_3 media after sterilisation through a 0.2 µm filter. Embryogenic callus (0.5 g) was cut into pieces of approximately 1.5 mm and immersed in the EMS containing media, sealed with parafilm and placed on a shaker for four hours at 70 rpm. Following incubation, callus was rinsed three times with 5 ml sterile water and air dried on sterile filter paper for 5 min in a laminar flow cabinet. Callus was then transferred to solid MS_3 media and incubated in the dark at 26°C for four weeks. Tissue was then transferred to MS media (without 2,4-D) and kept for six weeks under cool fluorescent light (Osram; 36W/640) with intensity of 50

μmol photons/m^2/sec at 26°C and a 16/8 hours light/dark regime for regeneration. Within the first week of regeneration, callus was covered with yellow cellophane filter layer to lower the light intensity and decrease light stress effects. After six weeks of regeneration, callus was transferred to larger containers containing MS media, to accommodate root formation. Statistical analyses were executed as described in *section 2.2.6.*

2.2.4. *In vitro* exposure of NCo310 to osmoticum selection agents

NCo310 sugarcane calli were placed on different concentrations of mannitol (Sigma- Aldrich, St. Louis, USA) and polyethylene glycol 6000 (PEG) (Merck, Darmstadt, Germany) to determine the suitable osmoticum as well as its respective optimal concentration to establish an efficient *in vitro* osmotic selection system.

2.2.4.1 Mannitol

Initially, three different concentrations of mannitol were tested for the effect it has on NCo310 sugarcane callus survival based on the method described by Errabii *et al.* (2007). These concentrations included 0, 100, 200 and 300 mM. However, after evaluation of callus induction and regeneration results additional, higher mannitol concentrations (400 mM, 700 mM and 1 M) were included.

For determining the minimal lethal concentration *in vitro*, 0.5 g embryogenic callus was cut into approximately 1.5 mm diameter pieces using a scalpel and placed on MS$_3$ media containing different mannitol concentrations. The cultures were then stored in the dark at 26°C for four weeks. After selection in the dark, callus was transferred to MS media and kept under cool white fluorescent light (Osram; 36W/640) with intensity of 50 μmol photons/m^2/sec at 26°C with a 16/8 hour light/dark regime for eight weeks for shoot formation. Callus was sub-cultured after every two weeks.

2.2.4.2 Polyethylene glycol

Initially, five different concentrations of PEG 6000 (0.5, 1, 1.5, 2 and 20% (w/v)) were investigated for the effect it has on NCo310 sugarcane callus survival, based on the method described by Wani *et al.* (2010). After evaluation of callus growth and regeneration, additional higher PEG 6000 concentrations (10, 15, 20 and 30% (w/v)) and longer PEG exposure periods described by Rao and Jabeen (2013) were included.

For *in vitro* osmotic tolerance selection, MS$_3$ media (MS media with 3 mg/L 2,4-D) was prepared and PEG added before autoclaving. Media containing 30% (w/v) PEG 6000 were prepared following the PEG-infused method described by Verslues and Bray (2004) to

overcome solidification problems. For this, 20 ml solid MS_3 media was poured in 100 mm x 20 mm plates and allowed to solidify. Once the media has solidified, 30 ml of liquid PEG media was overlaid on the solid media. The plates were then wrapped with parafilm to prevent the media from drying and stored for 12 to 15 hours in order for PEG to infuse into the solid media. Just before use, the liquid media was carefully poured off and the solid media with infused PEG was then used.

To determine the lethal PEG concentration for callus *in vitro*, 0.5 g of embryogenic NCo310 sugarcane calli were aseptically cut into very small pieces (1.5 mm diameter) (Figure 4A) using a scalpel and placed on prepared MS_3 selection media with previously described PEG concentrations. The petri dishes were sealed with micropore tape and stored in the dark at $26^{\circ}C$ for 4 (experiment 1) or 8 (experiment 2) weeks. For the third experiment (experiment 3), 0.5 g of calli (approximately 1.5 mm diameter) (Figure 4B) were placed in 15 ml liquid MS_3 media containing 10, 15, 20 or 30% (w/v) PEG for eight weeks in the dark at $26^{\circ}C$. These liquid callus cultures were sub-cultured weekly, cell clumps were allowed to settle and 5 ml old media was replaced with 7 ml of fresh liquid media.

For experiment 1, after four weeks on PEG media in the dark, calli were aseptically transferred to MS media (without 2,4-D) and stored under cool white fluorescent light at $26^{\circ}C$ with a 16/8 hour light/dark regime for further eight weeks for shoot regeneration. Callus was first cultured under yellow cellophane filter layer for the first week of regeneration in order to decrease light stress effects and then sub-cultured every two weeks onto fresh MS media.

For experiment 2, eight weeks of PEG selection on high concentrations (10, 15, 20 and 30% (w/v)) in the dark was followed by transfer of calli to MS media and stored under cool white fluorescent light at $26^{\circ}C$ with a 16/8 hour light/dark regime for osmotic stress recovery. Similar to experiment 1, callus was placed under a yellow cellophane filter layer to lower the light intensity for the first week of recovery. After two weeks of recovery, calli were transferred back to MS media containing respective PEG concentrations and kept under a 16/8 hours light/dark regime at $26^{\circ}C$ for nine weeks for shoot regeneration. Cultures were sub-cultured fortnightly.

For experiment 3, after eight weeks of liquid PEG selection in the dark, callus was aseptically transferred to solid MS media and stored under cool white fluorescent light at $26^{\circ}C$ with a 16/8 hour light/dark regime for four weeks for osmotic stress recovery. Similar to experiments 1 and 2, the callus was placed under yellow cellophane filter layer for the first week of recovery. After four weeks of recovery, callus was transferred to solid MS media with respective PEG concentrations for a further nine weeks and kept under cool white

fluorescent light at 26°C for shoot formation. Cultures were sub-cultured on a fortnight basis. Statistical analyses were executed as described in section *2.2.6.*

2.2.5. Mutagenesis of sugarcane callus using EMS and *in vitro* selection of osmoticum tolerant clones on PEG

Embryogenic calli (0.2 g) from cultivars NCo310, NCo376 and N19, were cut into approximately 1.5 mm diameter pieces and immersed in respective liquid 2,4-D media with 16 mM EMS. The tubes were sealed with parafilm and placed on a shaker for four hours at 70 rpm. Following the incubation period, callus was rinsed three times with 5 ml sterile water and air-dried on sterile filter paper for 5 min.

Mutagenised calli were transferred to petri dishes containing solid MS media with respective 2,4-D concentrations and 20% (w/v) PEG 6000. Cultures were kept in the dark at 26°C for eight weeks and sub-cultured every two weeks. After eight weeks, calli were transferred to MS media, without 2,4-D and PEG, and placed in the light under cool white fluorescent light (Osram; 36W/640) with intensity of 50 μmol photons/m^2/sec at 26°C with a 16/8 hour light/dark regime for two weeks of osmotic stress recovery. Calli were covered with yellow cellophane filter layer during the first week of recovery. Following recovery, calli were transferred back to MS selection media containing 20% (w/v) PEG (without 2,4-D) for ten weeks under the afore-mentioned light conditions for regeneration and shoot formation. Clones that survived the selection were identified and transferred to small jars with basic MS media to root and multiply and eventually be *ex vitro* acclimatised. Negative controls represent wildtype calli from each cultivar that had been cultured on PEG without exposure to EMS. Statistical analyses were carried out as for all cultivar experiments as described below.

2.2.6. Statistical analyses

For statistical analyses of callus size and shoot length across all EMS/PEG experiments, the treatments were randomised, with two replicates (two plates of 0.5 g callus for each PEG concentration). Calli size was quantified by measuring the callus cover area (mm^2) using isometric paper. Data was analysed for analysis of variance (1-way ANOVA) using the Daniel's XL Toolbox (v 6.60, Free Software Foundation Inc., Boston, USA). A modified version of the Levene's test described by Glantz and Slinker (2001) was first used to determine if there was equal variance across all treatments ($p > 0.05$). Secondly, the interaction between and within the treatments were tested for significance ($p \leq 0.05$), and

lastly, the effect or significance of each treatment were then determined by the least significant difference (LSD) using the Bonferroni-Holm Post hoc test (Daniels XL Toolbox, v 6.60, Free Software Foundation Inc., Boston, USA).

2.2.7. Preliminary drought pot trials

After *in vitro* mutagenesis and osmotic selection, clones of developed plantlets for each cultivar, as well as NCo310 plantlets, which served as controls, were hardened-off and prepared for *ex vitro* drought trials in the glasshouse. Approximately 2 kg of homologous soil mixture consisting of 2:1:1 parts palm peat:vermiculite:sand were placed in 20 cm pots. The plantlets were hardened-off and fertilised with 3 g/L Hygrotech and 2.5 g/L calcium nitrate (Hygrotech, Stellenbosch, South Africa) after two weeks in the glasshouse. Four week old glasshouse plants were then used for preliminary glasshouse drought pot trials.

At the beginning of the drought trial, all plants were saturated with 1 L of fertilised water and the soil moisture content was measured four hours after saturation. During the drought trial the soil moisture content was measured every morning before 09:00, whilst phenotypic changes were assessed on days 0, 5, 10, 12, 15, 17, 19, 22, 24, 27 and 30 after water was withheld from the plants. EMS treated plants that outlived the NCo310 controls, or were able to recover when re-watered after the drought stressed period, were considered as potentially drought tolerant.

Mutant plant clones that demonstrated drought tolerance during preliminary drought trials were subjected to follow-up drought trials in the glasshouse. Clones of potentially drought tolerant plants were multiplied *in vitro* on basic MS media. For the follow-up drought pot trials, three biological repeats of each mutant plant that showed potential drought tolerance, together with NCo310 control plants were subjected to a second 30 day follow-up drought trial. The drought trial protocol proceeded as in the preliminary drought trial described above.

2.3 Results

2.3.1. Establishing *in vitro* growth conditions for three sugarcane cultivars

2.3.1.1 Callus induction and regeneration

Calli induced from leave disks initiated from the different sugarcane cultivars and placed on different 2,4-D concentrations were visually assessed after six weeks of growth in dark at 26°C (Figure 2.1). Based on these observations, N19 callus induction was optimal at 1 mg/L

2,4-D and lowest at 4 mg/L 2,4-D, while NCo376 callus showed optimal callus formation at 3 mg/L with limited callus formation at 0.5 mg/L 2,4-D. N40 showed highest and lowest callus induction at 1 mg/L and 4 mg/L 2,4-D concentrations respectively (Table 2.1 and Figure 2.1).

Shoot formation was optimal for callus induced on 0.5, 1 and 2 mg/L 2,4-D concentrations for cultivars N19, NCo376 and N40 respectively (Figure 2.2). Overall, the *in vitro* coeffiency was optimal on 0.5, 1 and 2 mg/L 2,4-D for N19, NCo376 and N40, respectively (Table 2.1). When comparing the performance of cultivars *in vitro*, N40 performed the worst while NCo376 performed the best (Table 2.1; Figure 2.1 and 2.2). Rooting for all the cultivars occurred spontaneously on basic MS media without addition of any hormones (Figure 2.3).

Table 2.1. *In vitro* callus induction and shoot formation of three sugarcane cultivars placed on five different 2,4-D concentrations. Numerical values indicate the callus induction frequency (C), embryogenicity frequency (E), plantlet formation frequency (S) and average shoot length (L). The *in vitro* coefficient (Coef) adapted from Miroshnichenko *et al.* (2013), was calculated from three replicated culture plates containing 10 leaf disks each. The number of phenotypically off-type (white) shoots was also recorded.

Cultivar	2,4-D mg/L	C	E	S	L (cm)	Coef	Off-type shoot number
N19	0.5	60 ± 0.01	6 ± 0.1	3.53 ± 0.21	4.25 ± 0.12	5.13	5.33 ± 0.13
	1	80 ± 0.00	1 ± 0.00	3.17 ± 0.19	3.00 ± 0.13	0.68	7.67 ± 0.19
	2	77 ± 0.05	13 ± 0.5	0.90 ± 0.22	0.25 ± 0.01	0.45	0
	3	47 ± 0.02	43 ± 0.3	0.87 ± 0.42	0.50 ± 0.02	0.87	0
	4	20 ± 0.05	42 ± 1.3	0.87 ± 0.40	0.50 ± 0.02	0.37	0
NCo376	0.5	0	0	0	0	0	0
	1	30 ± 0.06	57 ± 3.0	1.90 ± 0.98	3.25 ± 0.12	10.5	0.17 ± 0.09
	2	67 ± 0.18	2 ± 0.1	0.70 ± 0.31	0.55 ± 0.02	0.09	0.10 ± 0.04
	3	100 ± 0.00	6 ± 0.1	2.07 ± 0.37	1.55 ± 0.06	1.96	0.53 ± 0.02
	4	83 ± 0.07	33 ± 1.8	0.03 ± 0.02	0	0	0
N40	0.5	17 ± 0.09	0	0	0	0	0
	1	73 ± 0.02	12 ± 0.1	1.2 ± 0.11	0.25 ± 0.01	0.26	0
	2	17 ± 0.05	49 ± 1.4	0.40 ± 0.13	3.50 ± 0.11	1.16	0
	3	20 ± 0.11	28 ± 1.5	0.67 ± 0.04	0.25 ± 0.01	0.01	0
	4	7 ± 0.04	0	0	0	0	0
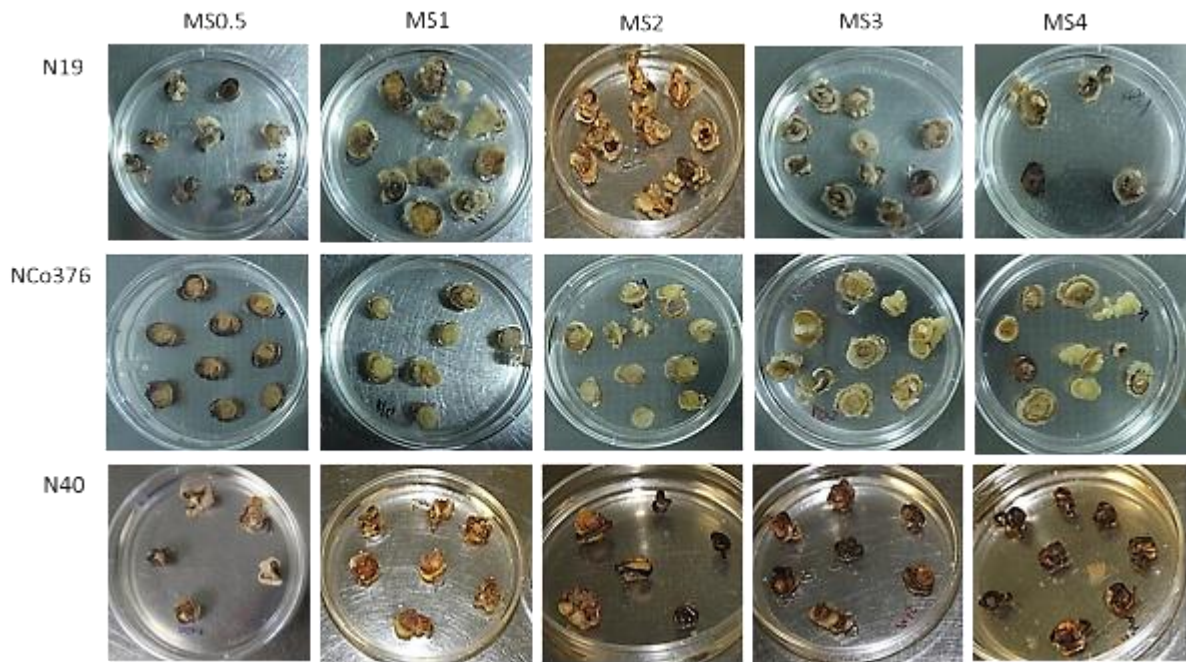
Figure 2.1: Callus induction from leaf discs cut from cultivars N19, NCo376 and N40 after eight weeks on MS medium containing different concentrations of 2,4-D. $MS_{0.5}$ to MS_4 indicate 0.5 to 4 mg/l 2,4-D respectively. The optimal auxin concentration for N19, NCo376 and N40 callus induction was determined as 3, 1 and 1 mg/L 2,4-D, respectively.
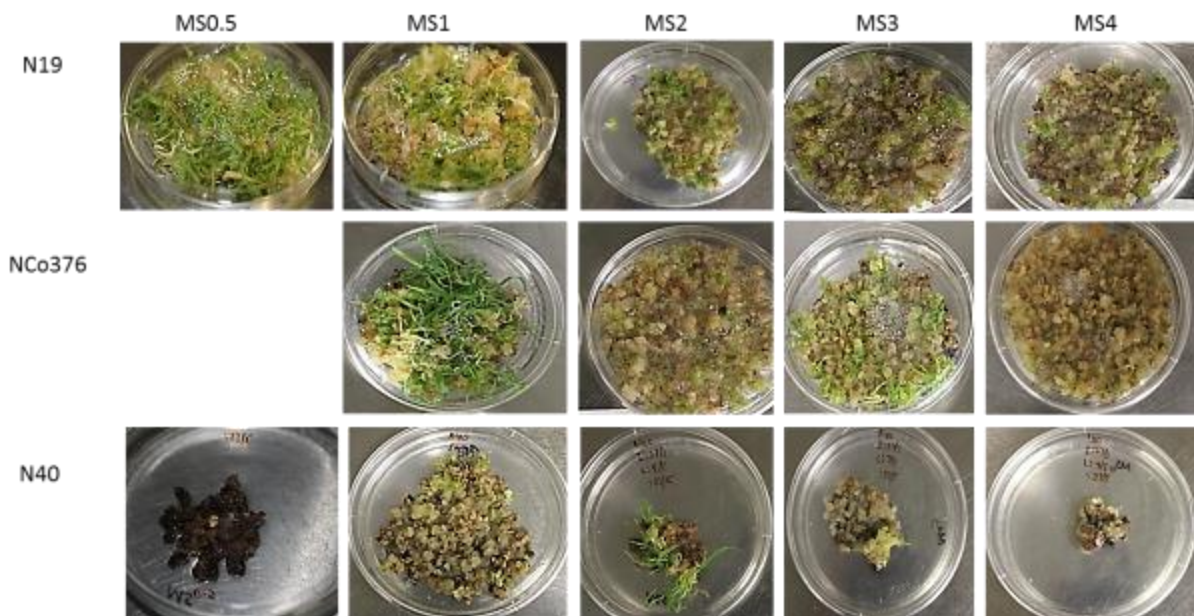
Figure 2.2: Shoot regeneration of cultivars N19, NCo376 and N40 after 10 weeks of regeneration and shoot formation on MS medium without 2,4-D. Open picture panes indicate that no tissue regeneration occurred. Optimal auxin concentrations for N19, NCo376 and N40 regeneration and shoot formation were determined as 1, 0.5 and 2 mg/L 2,4-D, respectively.
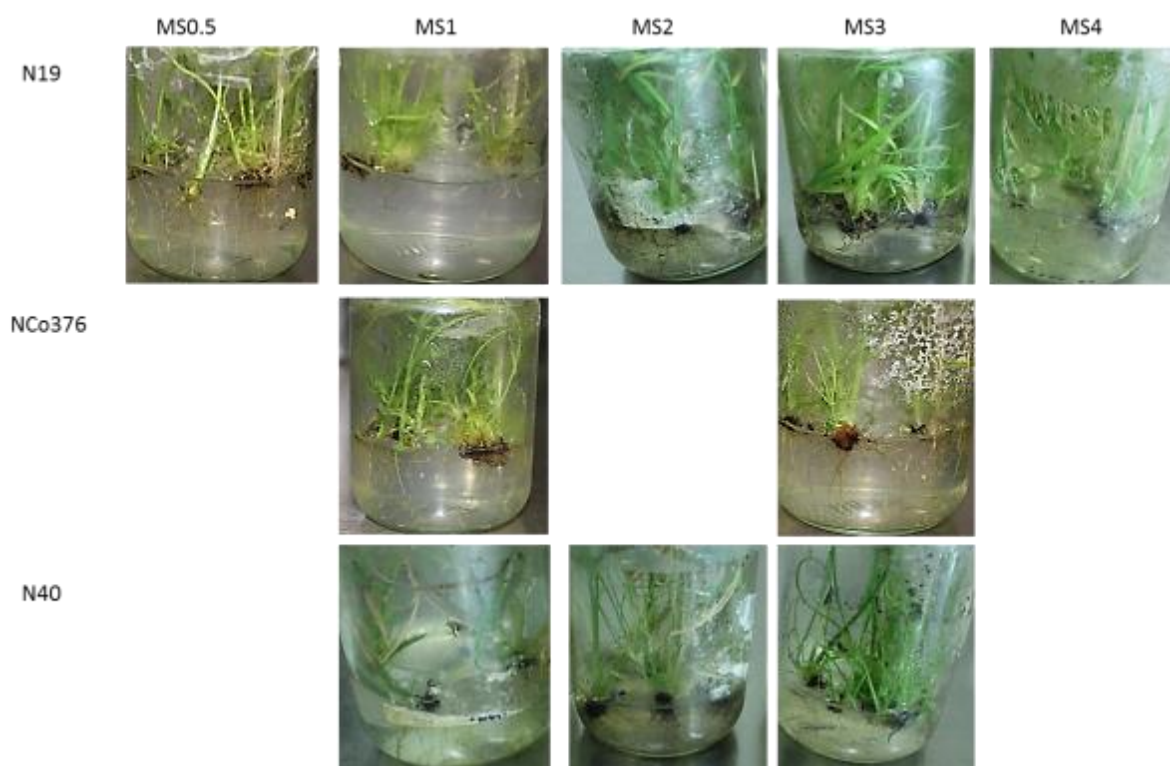
Figure 2.3: Rooting of plantlets from cultivars N19, NCo376 and N40 on MS medium without 2,4-D. Open picture planes indicate lack of tissue regeneration.

2.3.2. Establishing the optimal EMS concentration for mutation induction under *in vitro* conditions in Nco310 sugarcane callus

NCo310 sugarcane calli (three biological repeats of 0.2 g callus per treatment) exposed to different EMS concentrations were visually assessed for callus clump size, number of induced calli and the abundance of green and white (phenotypically off-type) shoots as well as shoot length.

After four weeks on MS_3 media, all exposed calli demonstrated considerable growth when compared to the initial callus clump size (Figure 2.4 A). However, calli exposed to lower EMS concentrations (5, 10 and 20 mM) had more callus clumps when compared to the control. Calli exposed to 25 and 50 mM EMS had fewer callus clumps compared to the control (Figure 2.5 A and 2.6). Exposure of calli to 25 mM EMS resulted in the least callus clumps across all concentrations (Figure 2.5 A and 2.6).

After ten weeks of regeneration and shoot formation, calli exposed to all EMS concentrations except 20 and 25 mM, showed no observable differences when compared to the wildtype

control. A very limited amount of phenotypically off-type shoots were observed in control callus as well as calli exposed to 5, 10 and 50 mM EMS (Figure 2.5 B and C). Callus exposed to 25 mM EMS showed the least regeneration of green shoots and the highest number of phenotypically off-type shoots compared to other concentrations (Figure 2.5 B). While callus exposed to 20 mM EMS showed highest green shoot regeneration and least chlorophyll deficient shoots.

Shoot length was highest in wildtype control callus (25 – 95 mm) and in callus exposed to 20 mM EMS (35 – 70 mm), moderate shoot length (15 – 55 mm) was observed in calli exposed to 10 and 25 mM EMS, and limited shoot elongation (10 – 15 mm) was observed in calli exposed to 5 and 50 mM EMS (Figure 2.5 C).

Callus growth and plantlet development on the lower concentrations of EMS (5 to 20 mM) was not significantly different from that of the control treatment. However, a significant reduction in callus growth after four weeks was seen in callus treated with 25 mM EMS compared to the control. Treatment with 25 mM EMS also resulted in the formation of phenotypically abnormal plants (such as chlorophyll deficient shoots) not seen in the lower concentrations and control treatments. Treatment with the highest tested EMS concentration (50 mM) resulted in plantlets that were unable to elongate and remained stunted at around 10 to 15 mm. From the current results, 20 mM EMS was considered the highest viable concentration for treatment of sugarcane callus because callus growth, as well as somatic embryo and plantlet development were normal and did not differ significantly from the untreated control

In this study, the ideal EMS concentration was that which generated useful mutations, without excessive killing of callus cells and/or inhibition of embryo germination, while still allowing the regeneration of a sufficiently large number of phenotypically normal plantlets. Therefore, based on this observation and the 16 mM EMS concentration recommended and reported by Koch *et al.* (2012) for their sugarcane mutagenesis experimentation, exposure to 16 mM EMS for four hours was applied in all further downstream experimentation in this study.
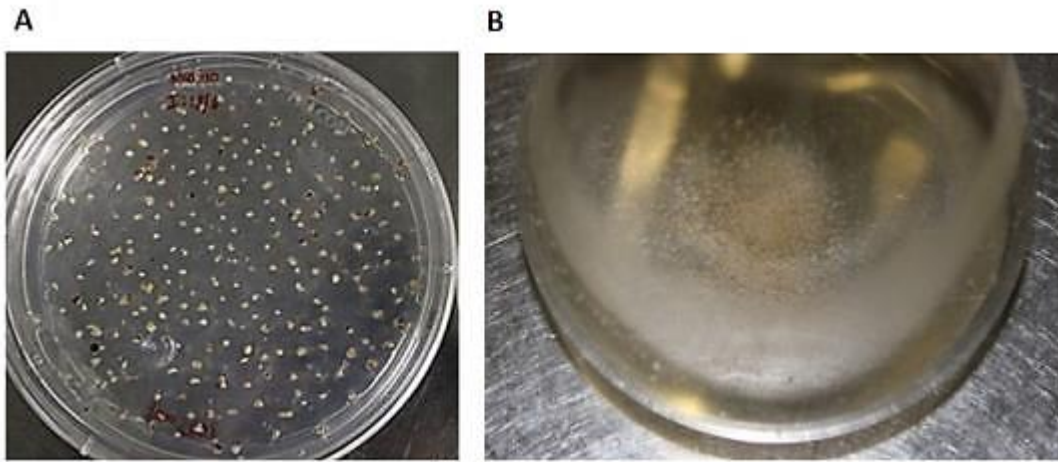
Figure 2.4: An illustration of the initial size of callus clumps placed on solid media (A) or liquid media (B) used for mutagenesis and osmotic selection experiments. Bar scale = 5 mm.
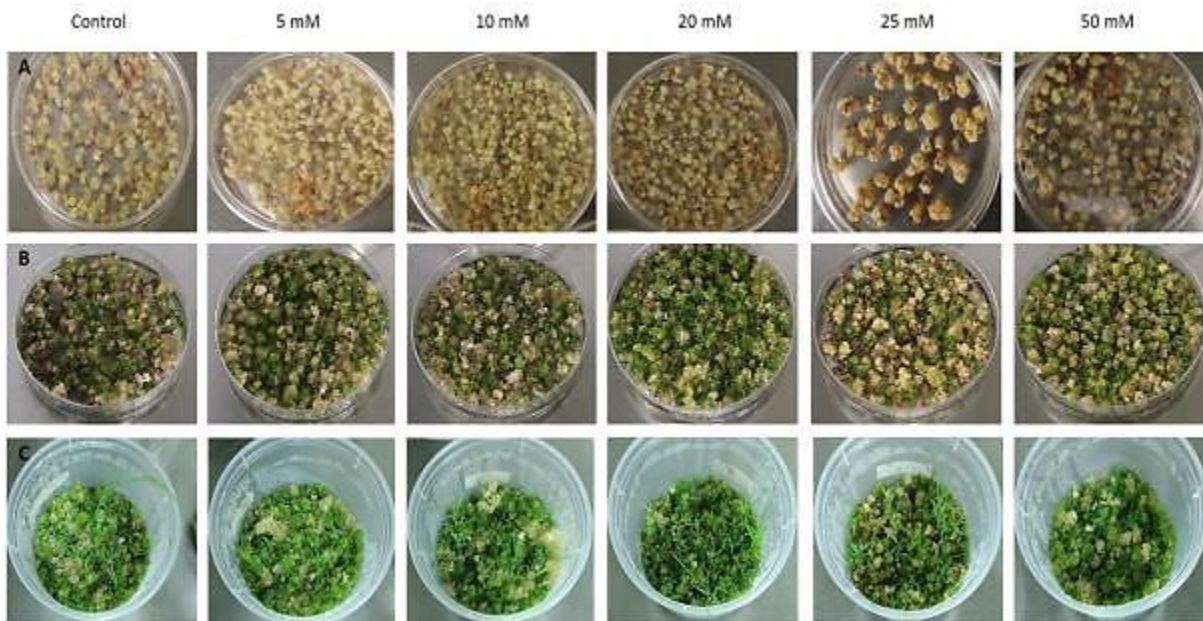
Figure 2.5: NCo310 callus exposed to different EMS concentrations (5 to 50 mM) cultured on medium containing 3 mg/L 2,4-D after four weeks in the dark (A); after six weeks of regeneration (B); and four weeks of shoot development (C) in the light on medium without 2,4-D.
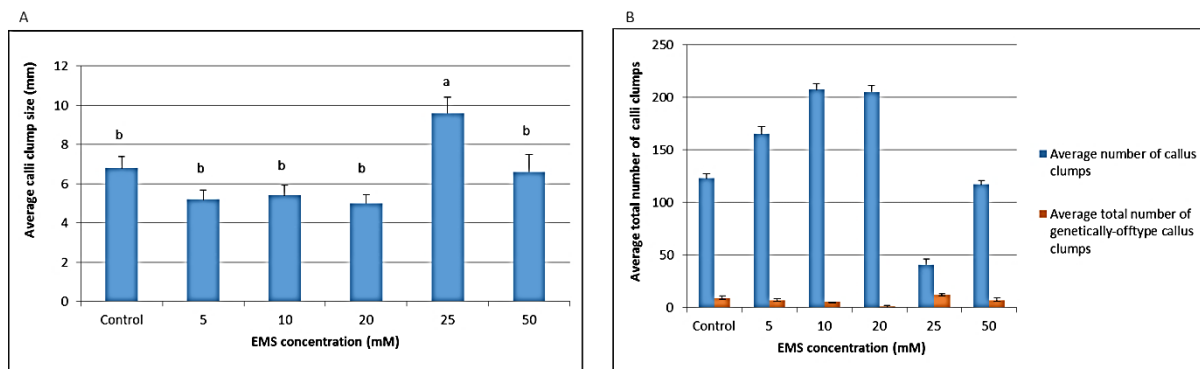
Figure 2.6: Bar graphs demonstrating; A) average callus clump size and; B) total number of callus clumps as well as the total number of off-type callus clumps observed on the different EMS concentration treatments. Significant differences were measured at $p > 0.05$, same superscript letters illustrate no significant difference observed. Error bars represent standard error of mean (SE). n=10 for average calli clump size and n=2 for average total number of calli clumps.

2.3.3. Exposing NCo310 sugarcane callus to PEG and mannitol to determine the optimal concentration and appropriate osmoticum for *in vitro* osmotic selection

2.3.3.1 Callus exposure to mannitol

After callus was exposure for four weeks to low mannitol concentrations in the dark, callus necrosis, growth and size were monitored. Calli clumps exposed to all mannitol concentrations as well as the wildtype control demonstrated considerable growth when compared to the initial size (Figure 2.4A) of the cultured callus clumps (Figure 2.8 A). When compared to the control callus, calli exposed to all mannitol concentrations showed no callus necrosis and no significant difference in callus clump growth and number (Figure 2.7 and 2.8 A).

Callus initially exposed to the different mannitol concentrations and cultured further for eight weeks in the light on MS medium without 2,4-D and mannitol, showed no difference in regeneration and formation of green shoots compared to the control (Figures 2.8 B). Since calli exposed to these mannitol concentrations reacted similarly to the control treatment, none of the concentrations were suitable for selecting cells for osmotic tolerance *in vitro* and higher mannitol concentrations were therefore tested.

Following calli exposure to higher mannitol concentrations for four weeks, callus clumps exposed to 400 mM mannitol was not significantly different in size to those of the wildtype control (Figure 2.9 A). Calli clumps exposed to 700 mM and 1 M mannitol, demonstrated

significantly smaller clump sizes when compared to the control calli (Figures 2.9 A). The total amount of callus induced on 700 and 1 M mannitol, all showed noticeable cell necrosis and significantly reduced growth compared to the wildtype control (Figures 2.9 B and 2.10). However, high concentrations of mannitol might be toxic to cells, therefore mannitol was excluded as a suitable selection osmoticum for *in vitro* osmotic tolerance.

Figure 2.7: Graphs representing NCo310 average callus clump sizes (A) and the average number of callus clumps per mannitol concentration for duplicate cultures (0.2 g callus per treatment plate) (B) after four weeks under mannitol selection in the dark. Significant differences were measured at p > 0.05, same superscript letters illustrate no significant difference observed between treatments. Error bars represent standard error of mean (SE). n=10 for average callus clump size and n=2 for average total number of calli.
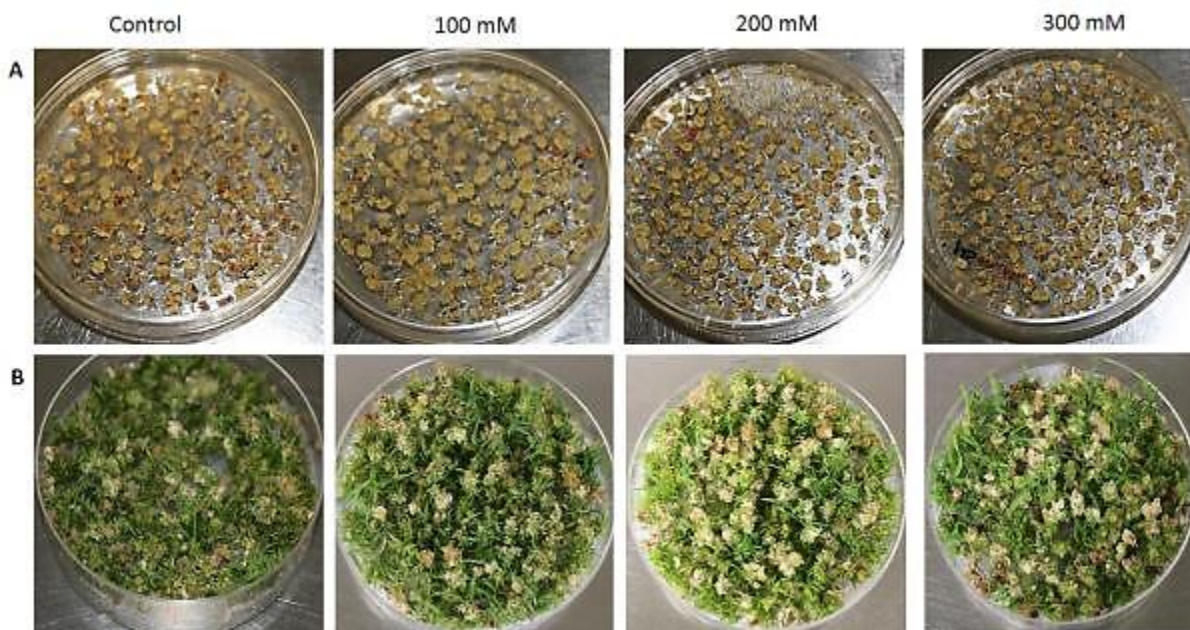
Figure 2.8: NCo310 callus exposed to 100, 200 and 300 mM mannitol for 4 weeks in the dark on MS$_3$ medium (A) and after 8 weeks of regeneration and shoot formation (B) in the light on MS medium without 2,4-D and mannitol. No noticeable differences were observed in callus growth after selection and after regeneration and shoot formation across all concentrations.
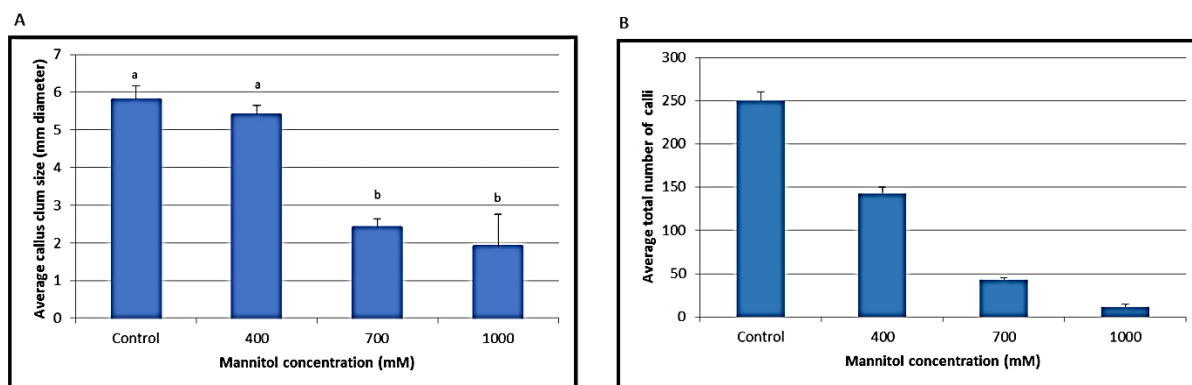
Figure 2.9: Graphs representing NCo310 average callus clump sizes (A) and the average number of callus clumps per mannitol concentration for duplicate cultures (0.2 g callus per treatment plate) (B) after four weeks under high mannitol selection in the dark. Regeneration and shoot formation were excluded from this experiment. Significant differences were measured at $p > 0.05$, same superscript letters illustrate no significant difference observed. Error bars represent standard error of mean (SE). n=10 for average callus clump size and n=2 for average total number of calli.
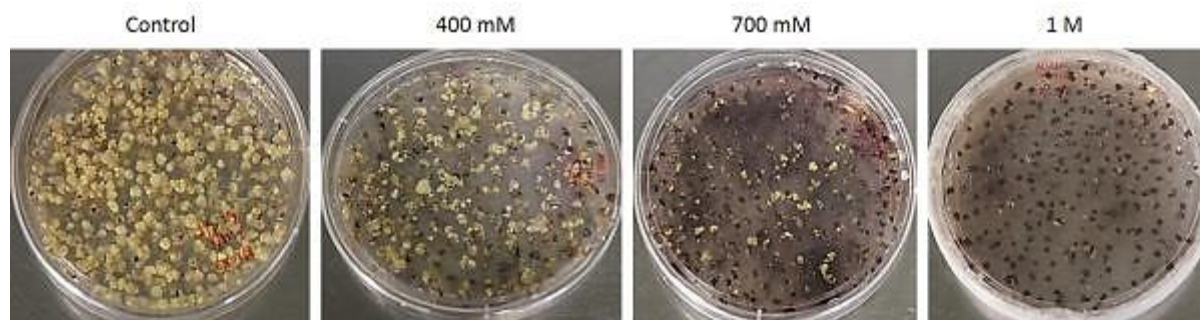
Figure 2.10: NCo310 callus exposed to 400, 700, and 1000 mM mannitol on MS$_3$ medium containing 3 mg/L 2,4-D after four weeks in the dark. Significant differences were observed in callus growth after selection.

2.3.3.2 Callus exposure to PEG

Experiment 1

Callus clump size and necrosis of callus exposed to different PEG concentrations after four weeks in the dark were assessed. Calli exposed to all PEG concentrations, excluding 20%

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(w/v) showed no significant difference in clump size when compared to the control treatment (Figure 2.11 A and 2.12 A). Correspondingly, the average number of callus clumps was lowest on 20% (w/v) PEG (Figure 2.12 B). In addition, callus exposed to 20% (w/v) PEG also demonstrated signs of cell necrosis, which were illustrated by colour change of calli from white to black (Figure 2.11 A).

After eight weeks in the light without PEG, calli regeneration and shoot formation were assessed. Callus exposed to 20% (w/v) PEG showed the least amount of green shoot regeneration compared to the control and all other concentrations (Figure 2.11 B). Four weeks of PEG exposure did not sufficiently suppress shoot formation even when calli was exposed to 20% (w/v) PEG and calli cells were able to re-gain osmotic balance sufficiently to regenerate shoots. No chlorophyll deficient white shoots were seen in the control and calli exposed to all PEG concentrations.

Figure 2.11: NCo310 callus exposed to different PEG concentrations on medium containing 3 mg/L 2,4-D after four weeks in the dark (A) and after eight weeks of regeneration and formation of shoots (B) in the light on MS medium without 2,4-D and PEG.
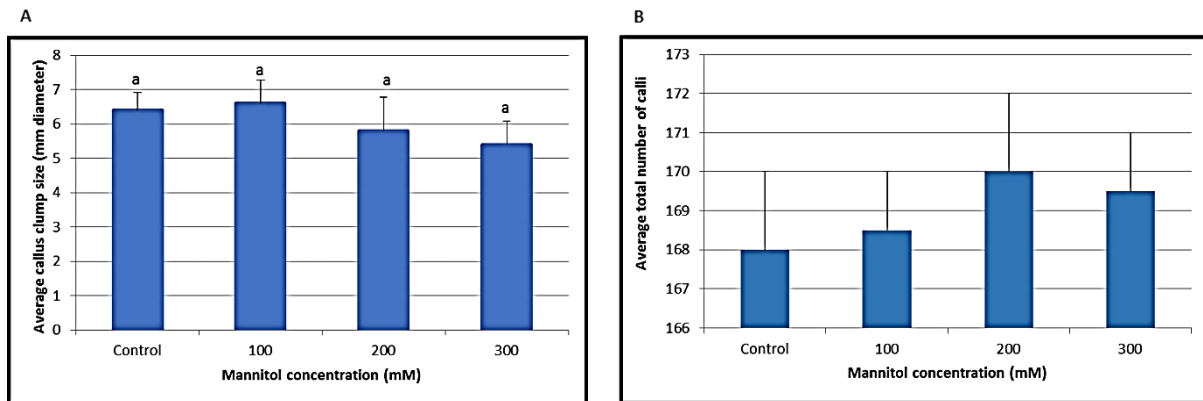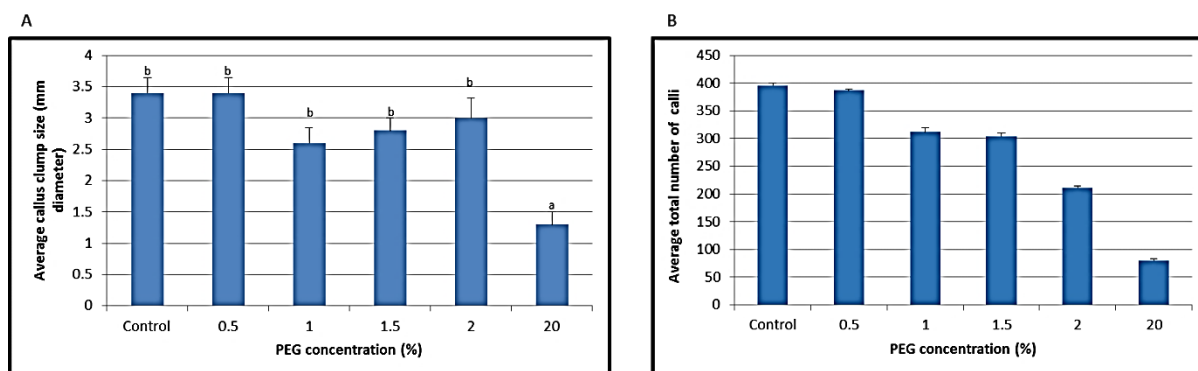
Figure 2.12: Graphs representing NCo310 average callus clump sizes (A) and the average number of callus clumps per PEG concentration for duplicate cultures (0.2 g callus per treatment plate) (B) after four weeks under PEG selection in the dark. Significant difference was measured at p > 0.05, same superscript letters illustrate no significant difference observed between different treatments. Error bars represent standard error of mean (SE). n=10 for average callus clump size and n=2 for average total number of calli.

Experiment 2

NCo310 callus was exposed to higher concentrations of PEG (10, 15, 20 and 30% (w/v)) for prolonged periods (Figure 2.13).

After PEG selection in the dark for eight weeks, calli clump size, number and death across all concentrations were assessed. Of the selected calli, calli clumps exposed to 20% (w/v) PEG demonstrated a significant reduction in growth when compared to the wildtype control (Figure 2.13 A and 2.14 A and B). Correspondingly, the average number of calli clumps per treatment was the least for callus exposed to 20% (w/v) PEG (Figure 2.14 B). Furthermore, 20% (w/v) PEG resulted in callus cells that changed colour from white to black, demonstrating necrosis (Figure 2.13 A).

Following PEG recovery, after two weeks under filtered light, calli previously cultured on 30% (w/v) PEG demonstrated a noticeable increase in growth and initial shoot development, while callus previously cultured on 20% (w/v) PEG showed the least growth and shoot development when compared to the control and all other treatments (Figure 2.13 C and 2.14 B).

Because of callus cell death, significantly reduced callus size and limited callus regeneration observed after the osmotic recovery phase, callus exposed to 20% (w/v) PEG was subjected to a further eight weeks of regeneration on basic MS media supplemented with 20% (w/v)

PEG in the light (Figure 2.13 C and 2.14 C). A significant reduction in callus that formed shoots was observed when compared to the control treatment. In the control, 100% organogenesis was observed while calli treated with 20% (w/v) PEG only resulted in approximately 45% organogenesis of which the further portion was unable to develop and elongate into functional shoots.

These results were supported by observations of experiment 1 and show that 20% (w/v) PEG results in the desired lethality and least regeneration of callus, and was, therefore, chosen as the optimal concentration for osmotic tolerance selection in further *in vitro* experiments. Furthermore, experiment 2, showed that extended time of PEG exposure was necessary to result in sufficient growth suppression of cultures.

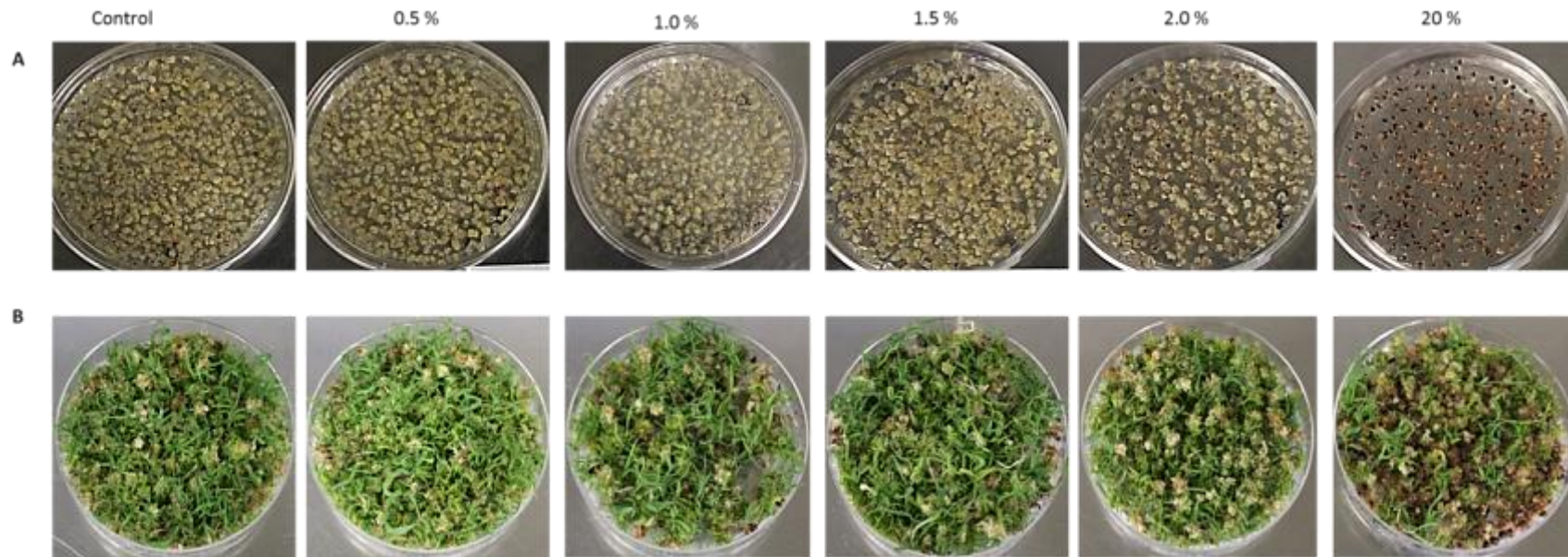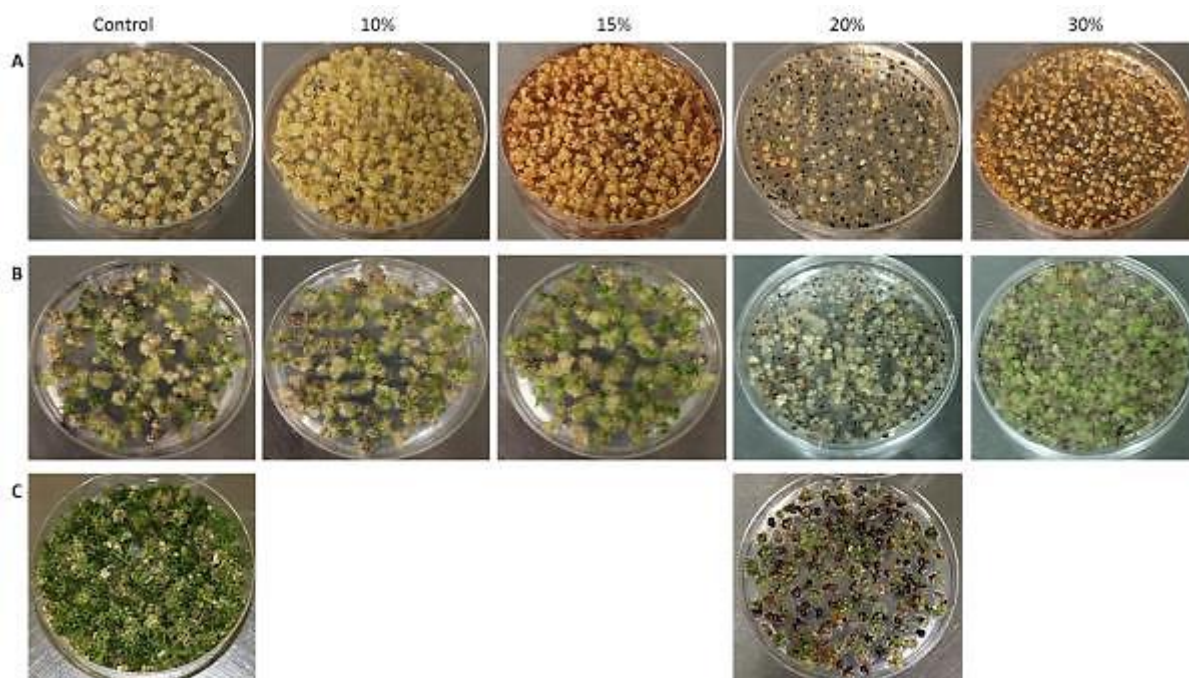**Figure 2.13:** NCo310 callus exposed to different PEG concentrations on medium containing 3 mg/L 2,4-D after 8 weeks in the dark (A); after 2 weeks of recovery in the light on MS medium without 2,4-D and PEG (B); and after 8 weeks of regeneration and shoot formation on MS medium containing 20% PEG concentration without 2,4-D (C).
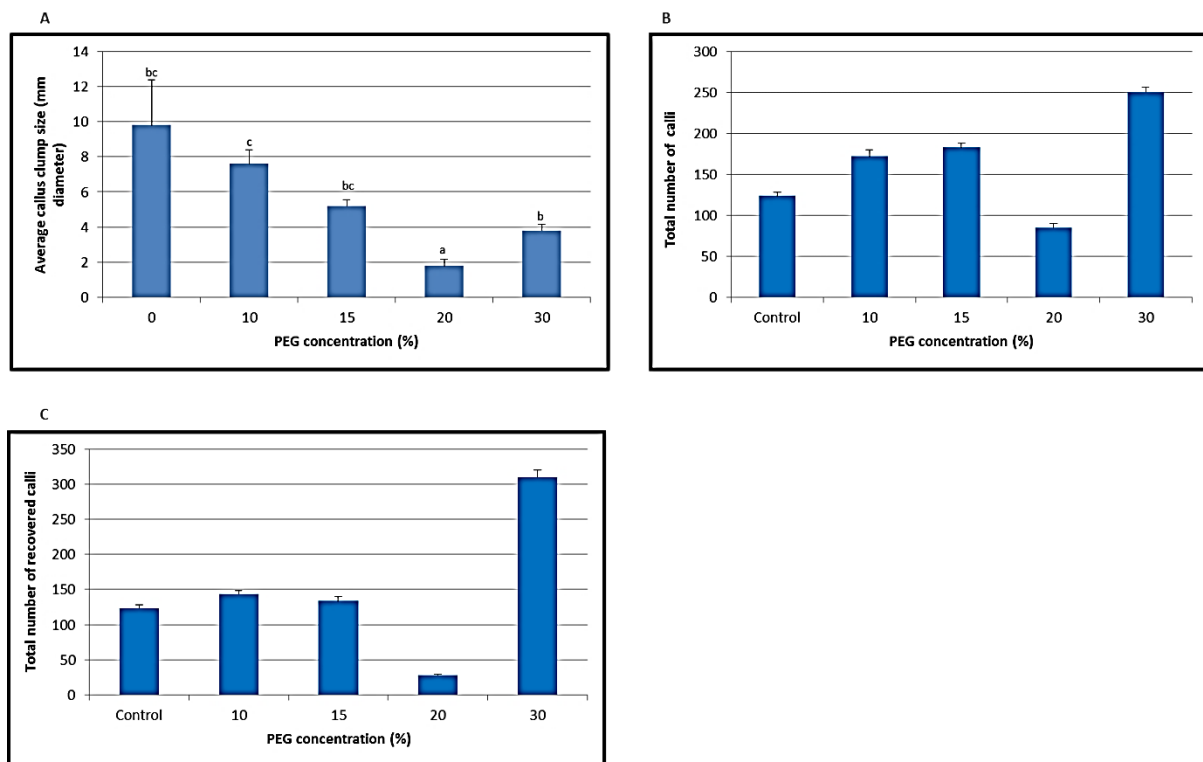
Figure 2.14: Graphs representing NCo310 average callus clump sizes (A) and the total number of proliferating callus clumps (B) for duplicate cultures (0.2 g callus per treatment plate) representing the same PEG concentration after eight weeks of PEG selection in the dark. After two weeks of PEG recovery in the light following the PEG stress, C represents the total number of recovered calli clumps. Concentrations with same subscript letters indicate no significant difference ($p > 0.05$) observed between the concentrations. Error bars represent standard error of the mean (SE). n=10 for average callus clump size and n=2 for average of total number of calli and recovered calli clumps.

Experiment 3

After eight weeks of PEG selection in liquid growth media in the dark, calli growth was visually assessed across all concentrations. When compared to the initial callus (Figure 2.4 B), all calli, except those exposed to 20 and 30% (w/v) PEG, demonstrated considerable growth (Figure 2.15 A). Callus exposed to 30% (w/v) PEG showed no growth when compared to the initial calli culture.

Figures 2.15 B, 2.16 A and B show the results of PEG recovered calli after four weeks in the light. Recovered callus clump size, number and necrosis characteristics were assessed. Compared to the control, calli exposed to 10 and 15% (w/v) (w/v) PEG showed no significant difference in terms of callus clump size and number (Figure 2.15 A). However, significant callus size and number reduction in callus exposed to 20% (w/v) (w/v) PEG was observed, while callus exposed to 30% (w/v) (w/v) PEG still showed no growth and was therefore

excluded from further experiments (Figure 2.15 B and 2.16 A). Interestingly though, no callus death was observed across all PEG concentrations at this stage.

After eight weeks of shoot formation on PEG selection in the light, assessment of growth was based on shoot abundance, proportion of green and white shoots as well as callus death. Callus exposed to 20% (w/v) (w/v) PEG was the only treatment that demonstrated the formation of considerably high amounts of phenotypically off-type (white) shoots. A noticeable reduction in shoot length and abundance was observed in calli exposed to 10, 15 and 20% (w/v) (w/v) PEG when compared to the control treatment (Figure 2.15 C and 2.16 C). Furthermore, callus exposed to 20% (w/v) PEG showed callus cell death (illustrated by black calli clumps) when compared to other concentrations including the control (Figure 2.15 C).

Noticeable variations in root length were observed in all treatments including the control after 8 weeks on PEG selection in the light (Figure 2.17). The longest roots were observed in control and 10% (w/v) PEG exposed plantlets (average root length of 70 mm). Moderate root lengths were observed in plantlets exposed to 15% (w/v) PEG (average of 15 mm) and the shortest roots in plantlets exposed to 20% (w/v) PEG (average length of 10 mm).

Overall, 20% (w/v) PEG treatments resulted in similar growth phenotypes in both solid and liquid growth media. The treatment demonstrates significantly less abundant green shoot formation as well as cell necrosis when compared to all other treatments, including the control and was therefore used in further experiments. Furthermore, when using liquid media cultured cells were easily contaminated with no possibility of recovery. Solid media was therefore preferred for selection.
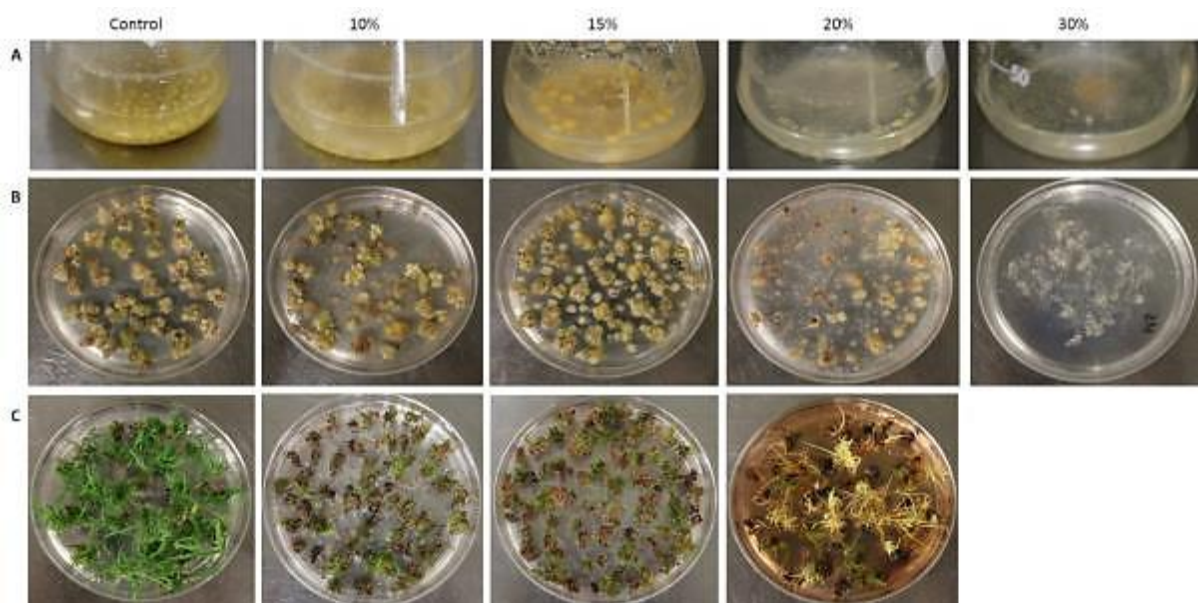
Figure 2.15: NCo310 callus grown in liquid medium containing 3 mg/L 2,4-D and different concentrations of PEG after eight weeks of selection in the dark (A); after four weeks of PEG stress recovery in the light on MS medium with neither 2,4-D nor PEG (B); and after eight further weeks of regeneration and shoot formation on MS medium containing respective PEG concentrations without 2,4-D (C). Open panels illustrate callus that were unable to grow or regenerate.
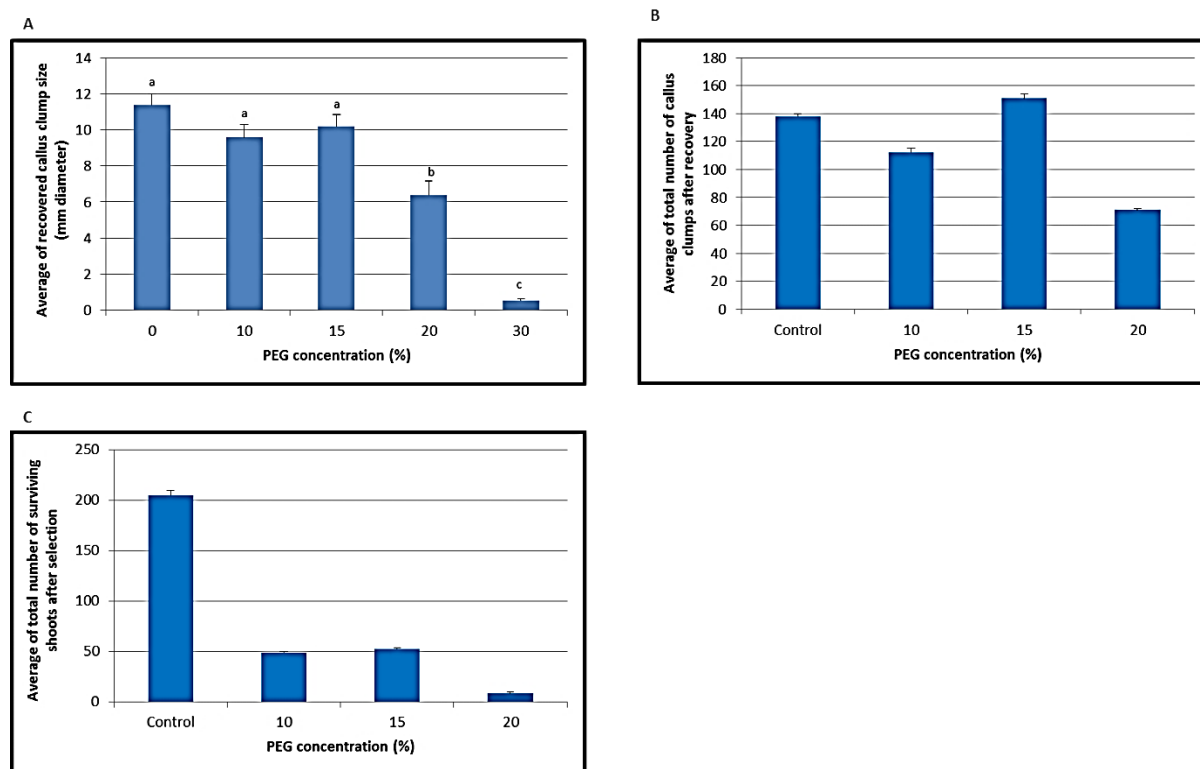
Figure 2.16: Graphs representing NCo310 average callus clump size (A) and the average number of callus clumps (B) per PEG concentration for duplicate cultures (0.2 g callus per treatment plate) after four weeks recovery in the light following the liquid PEG stress phase; after a further eight weeks of PEG selection in the light following the recovery phase, the average total number of surviving shoots was recorded (C). Significant difference was measured at $p > 0.05$, same superscript letters illustrate no significant difference observed between different treatments. Error bars represent standard error of mean (SE). n=10 for average of recovered callus clump size and n=2 for average of total number recovered calli clumps and surviving shoots.
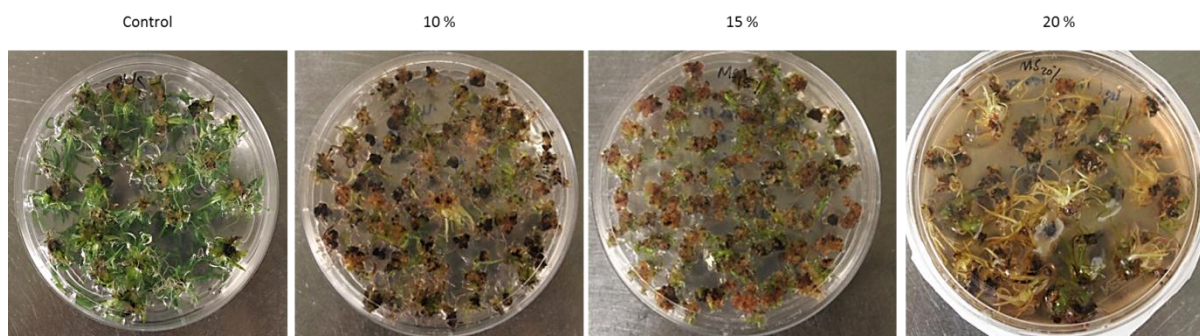
Figure 2.17: Root development of NCo310 callus initially grown in liquid medium, after eight weeks of regeneration and shoot formation on MS medium containing respective PEG concentrations without 2,4-D in the light. Longest roots were observed in the control and 10% (w/v) PEG exposed plantlets, followed by plantlets exposed to 15% (w/v) PEG. Shortest roots were observed in plantlets exposed to 20% (w/v) PEG.

2.3.4. Mutagenise callus from three sugarcane cultivars using the pre-determined EMS concentration followed by *in vitro* selection on PEG and *ex vitro* drought pot trials

2.3.4.1 Exposure of calli to EMS and in vitro PEG osmoticum selection

NCo310, NCo376 and N19 sugarcane calli were initiated on *in vitro* growth media containing 3, 1 or 0.5 mg/l 2,4-D respectively, exposed to 16 mM EMS and selected on 20% (w/v) PEG as pre-determined. Phenotypic differences such as callus growth (size and number), survival and shoot regeneration were monitored and assessed between the cultivars after each respective growth/selection phase (Figure 2.18).

Following culturing of EMS treated calli in the dark for eight weeks on MS medium supplemented with 20% (w/v) PEG, considerable callus growth was observed in wildtype control calli in all cultivars compared to the initial size cultured (Figure 2.4 A and 2.18 A), with NCo310 and N19 showing the highest and the lowest callus growth respectively (Figure 2.18 A). However, EMS treated calli growth was considerably reduced for all cultivars with N19 cultivar demonstrating the least growth (Figure 2.18 A and 2.19). Furthermore, highest and lowest callus necrosis was observed in N19 and NCo376 EMS treated calli respectively (Figure 2.18 A).

EMS treated and wildtype control calli were transferred to the light for two weeks on MS media with neither PEG nor 2,4-D for recovery (Figure 2.18 B). Amongst the control calli, all cultivars showed high levels of organogenesis, with the most pronounced regeneration observed in N19, followed by NCo310 and NCo376. However, amongst the EMS treated

calli, no distinctive differences were observed in terms of regeneration of green shoot buds. Compared to the wildtype, all cultivars resulted in limited embryogenicity, while EMS treated calli across all cultivars showed significant osmotic recovery illustrated by growth after two weeks on media lacking PEG (Figure 2.18 B and 2.19).

Recovered calli were then transferred to 20% (w/v) PEG MS media without 2,4-D for approximately 10 weeks in the light for further osmotic selection during shoot regeneration. In general, significantly fewer green shoots were observed in EMS treated calli across all cultivars when compared to their respective non-mutated controls. NCo376 EMS treated callus showed the least green shoots formation, followed by N19, while NCo310 EMS treated calli regenerated the highest number of green shoots (Figure 2.18 C and 2.19). Additionally, calli death was also observed in EMS treated calli across all cultivars. No callus or shoot survival was observed in the non-mutated callus exposed to 20% (w/v) PEG across all cultivars due to induced osmotic stress (Fig 2.18 D). Therefore the surviving EMS treated green shoots observed for the different cultivars most likely indicate osmotic tolerance and were therefore selected for further drought experiments. Green shoots that survived the *in vitro* osmotic selection were isolated, placed on MS media with neither 2,4-D nor PEG for root development, plantlet formation and multiplication for *ex vitro* drought pot trials (Figure 2.20).
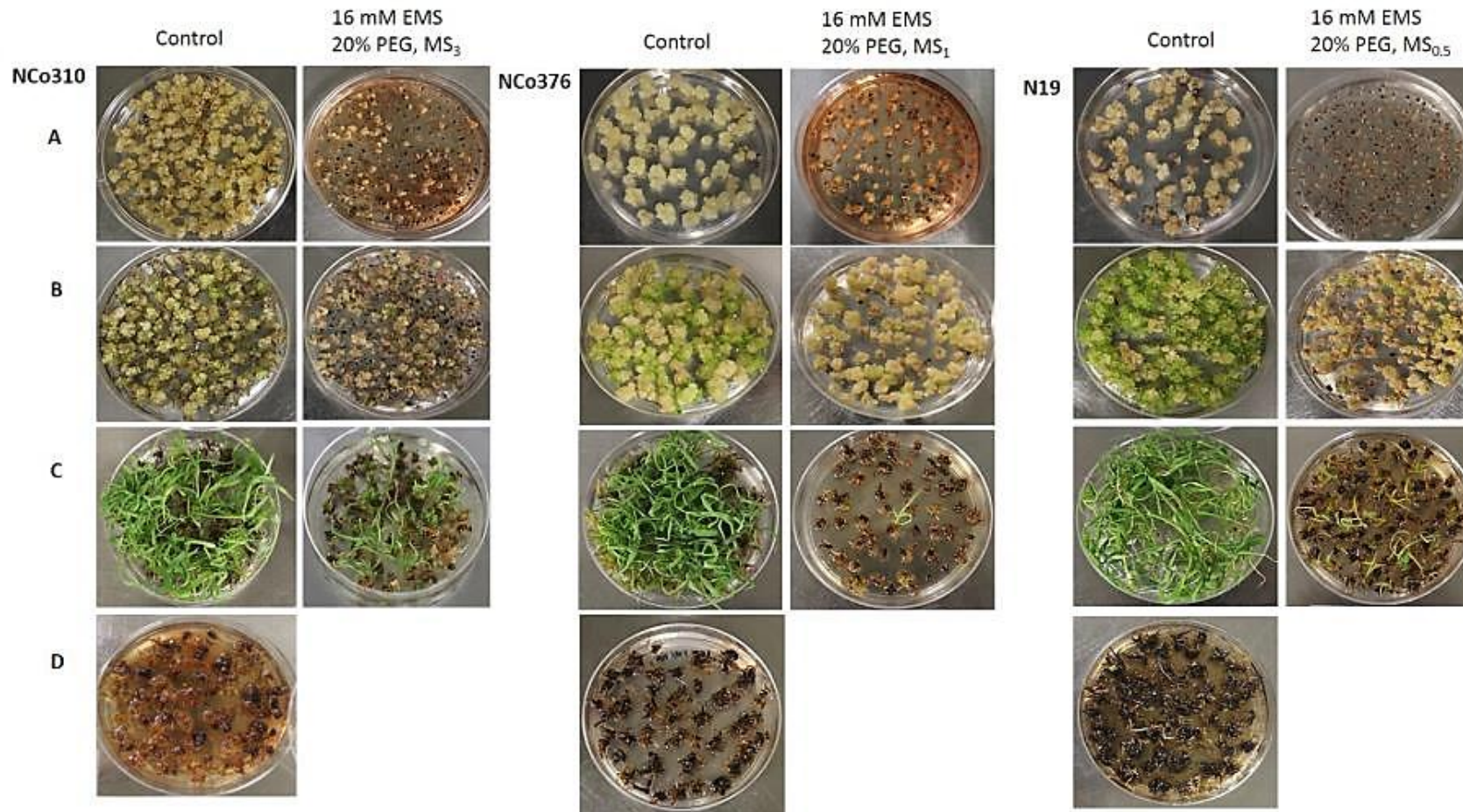
Figure 2.18: EMS treated and non-mutated calli from the different cultivars, NCo310, NCo376 and N19 after eight weeks in the dark on PEG selection media on their respective optimal 2,4-D concentrations (A); after two weeks in the light for recovery on media containing neither PEG nor 2,4-D (B); after 12 weeks of a further PEG selection following the recovery in the light without 2,4-D (C); and PEG controls after 14 weeks in the light, where calli were not EMS treated but cultured on the different growth media containing PEG (D).
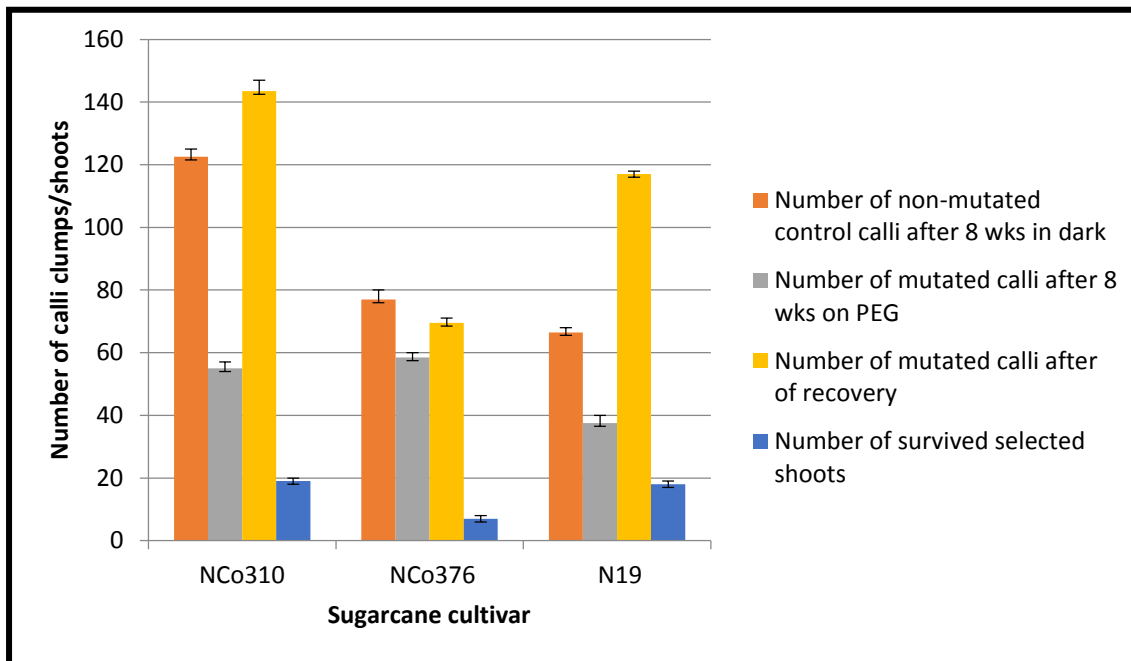
Figure 2.19: Calli growth for each cultivar after the different growth phases. The error bars demonstrate standard error of the means (SE). n=2
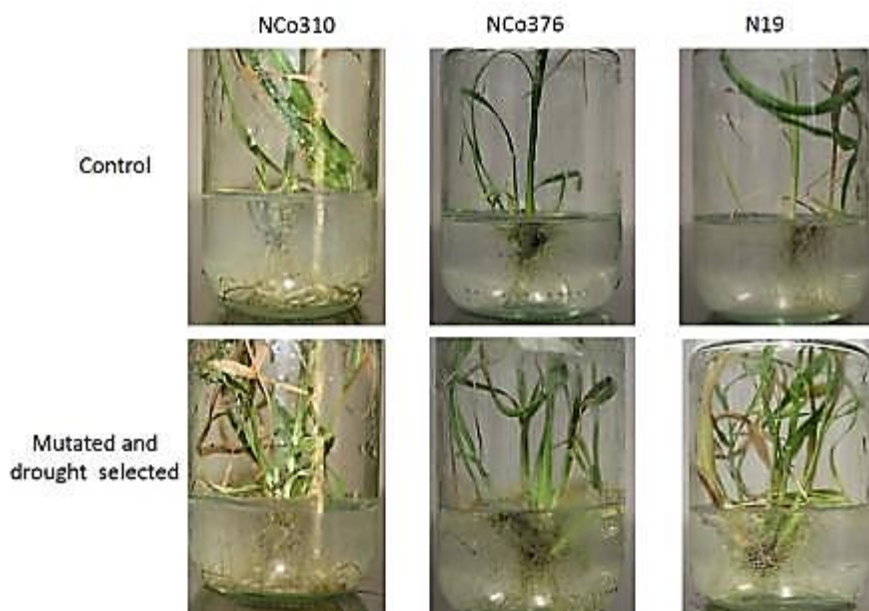
Figure 2.20: Rooting, plantlet development and multiplication of selected NCo310, NCo376 and N19 clones in the light on MS media containing neither PEG nor 2,4-D after mutation with EMS and osmotic selection on PEG.

2.3.4.2 Preliminary drought stress pot trials for NCo310 sugarcane cultivar

Drought stress pot trials were conducted only on NCo310 EMS treated clones as this information was not yet available for the NCo376 and N19 EMS treated clones since the preliminary drought trials are yet to be conducted.

<u>Pot trial 1</u>

Following the four week *ex vitro* acclimatisation, single biological copies of NCo310 selected lines were exposed to 30-35 days of preliminary pot drought trials, where the soil moisture content and phenotypic evidence of plant survival were assessed.

A uniform decrease in soil moisture content over time for all pots included in the trial was observed (Figure 2.21). For the first preliminary drought pot trial, two potential mutants, 310EP 4.4A and 4.5A, outlived the NCo310 control, which is more drought tolerant (SASRI communication). However, an additional EMS treated plant, 310EP 1.1C died at the same time as the NCo310 control (Figure 2.22). To further confirm drought tolerance in these three independent EMS treated lines, multiple clones (three) of each line were subjected to a follow-up drought pot trial (Figure 2.25 and Table 2.2).

Three days after plants were presumed dead, each plant was re-watered to observe the occurrence of possible plant recovery. In this regard one EMS treated plant, 310EP 4.1A demonstrated new shoot growth (Figure 2.22; green outline). Because of this line's interesting response to water deficit and re-watering, it was also included in the follow-up drought pot trial together with the three afore-mentioned selected mutant lines (Table 2.2).
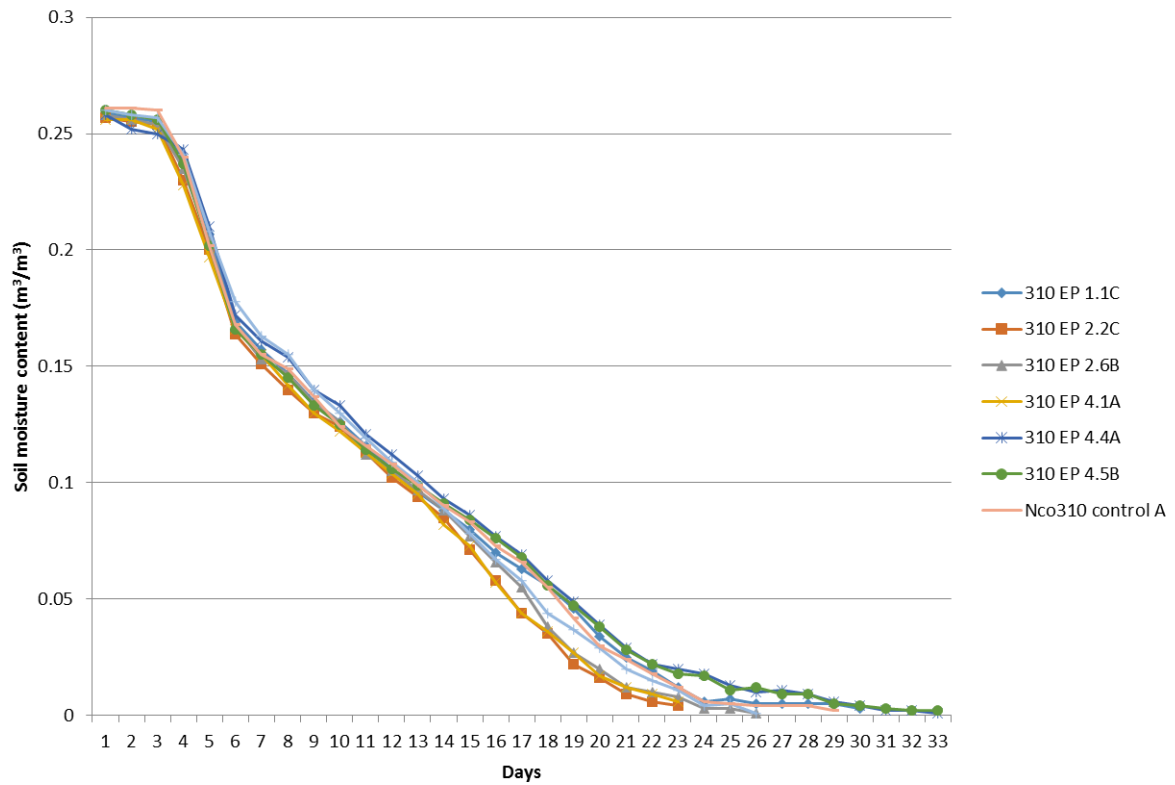
Figure 2.21: EMS treated NCo310 clones drought pot trial 1. A graph illustrating soil moisture content showing the uniform decrease in water content in each pot over time. NCo310 control represents the most drought tolerant cultivar that provides a threshold for potential drought tolerance in experimental lines.
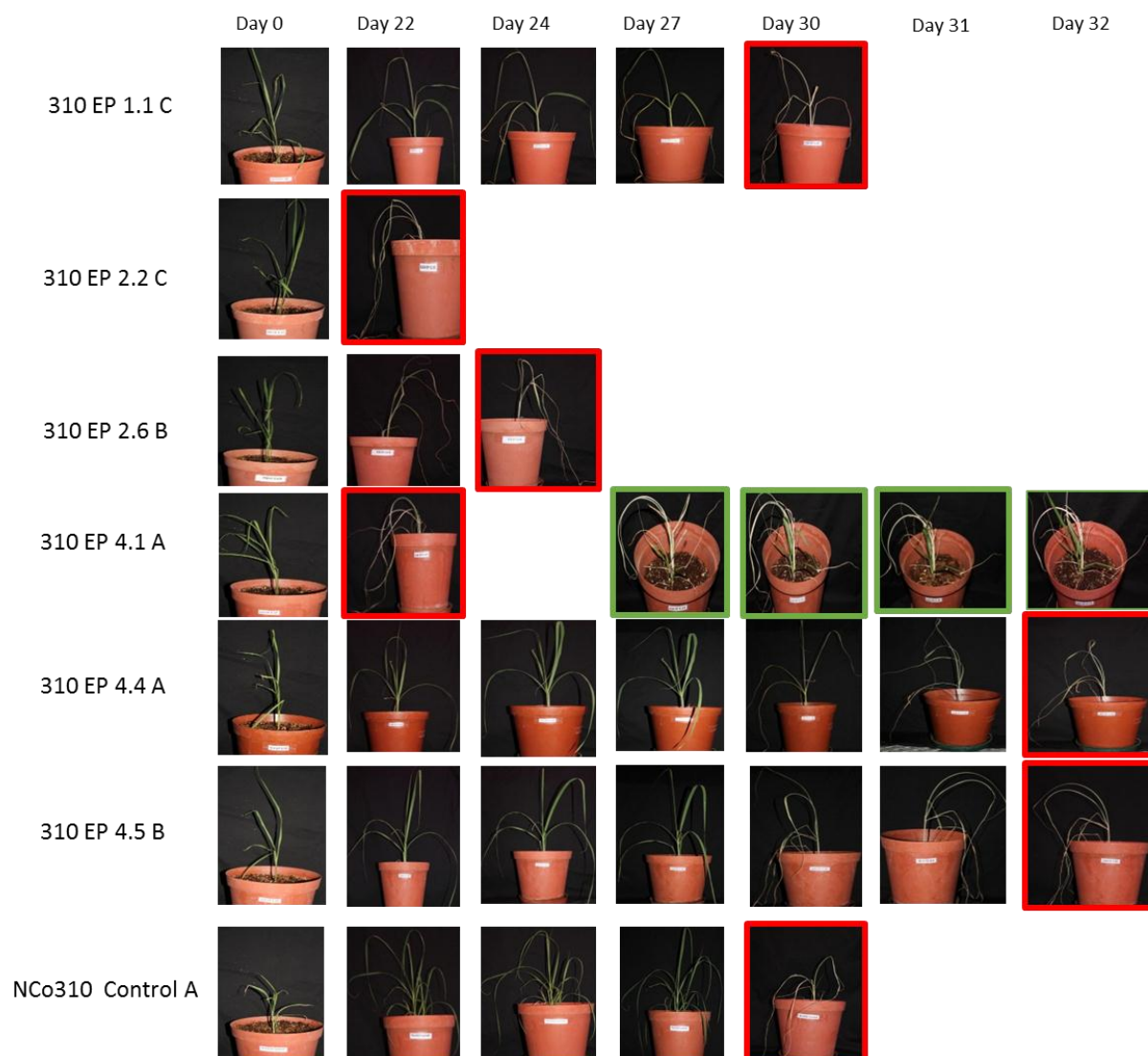
Figure 2.22: EMS treated NCo310 clones drought pot trial 1. Phenotypic monitoring of the first preliminary drought pot trials over time. Pictures outlined with red represent the day that the respective plant died, while pictures outlined with green represent re-watered plants recovering after water deficit.

Pot trial 2

Using different clones from the first preliminary drought trial, a second preliminary drought pot trial (Figure 2.24) was conducted. This included one biological copy of each EMS treated line as well as biological triplicates of the NCo310 wildtype controls. A uniform decrease in soil moisture content over time for all pots included in the trial was observed with the exception of one of the NCo310 controls (Figure 2.23). EMS treated plant 310EP 7.1A outlived all control plants and was therefore thought to be potentially drought tolerant. In

addition, EMS treated plants, 310EP 4.6A, 7.2A and 3.3A outlived one NCo310 control. Although they did not outlive all three control sets, they would also be considered for future drought tolerance experiments. Following the death of all plants, pots were re-watered to monitor recovery. However, no plant showed any signs of recovery.
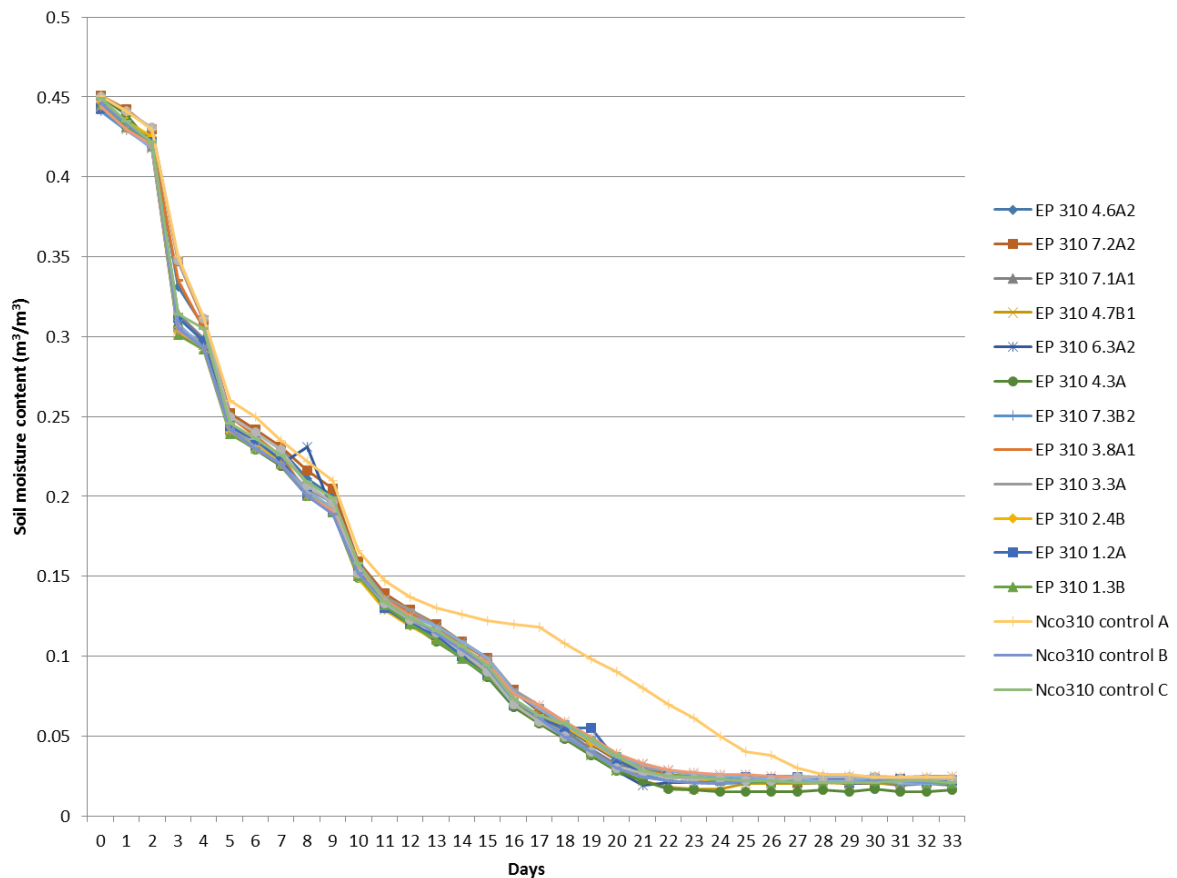
Figure 2.23: EMS treated NCo310 clones drought pot trial 2. A graph illustrating soil moisture content showing uniform decrease in water content in each pot over time. NCo310 control represents the most drought tolerant cultivar that provides a threshold for potential drought tolerance in experimented lines.
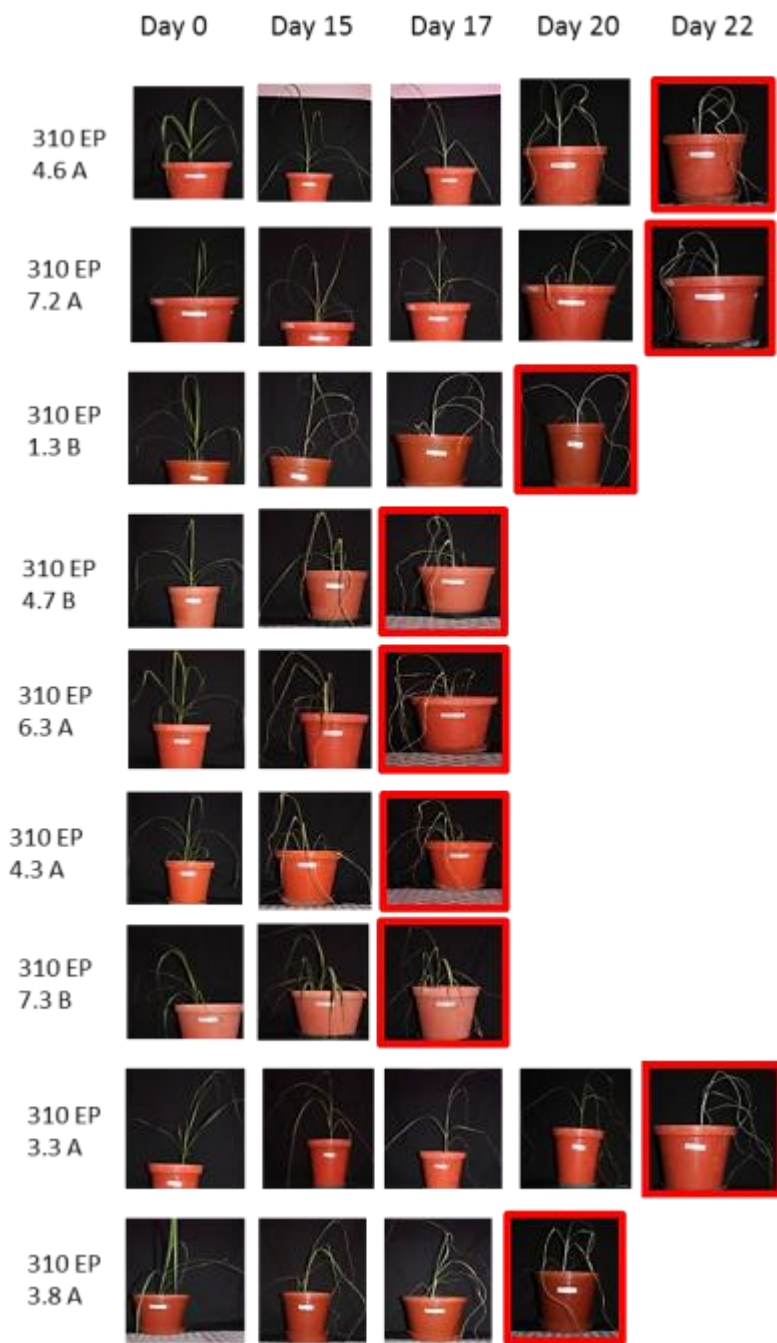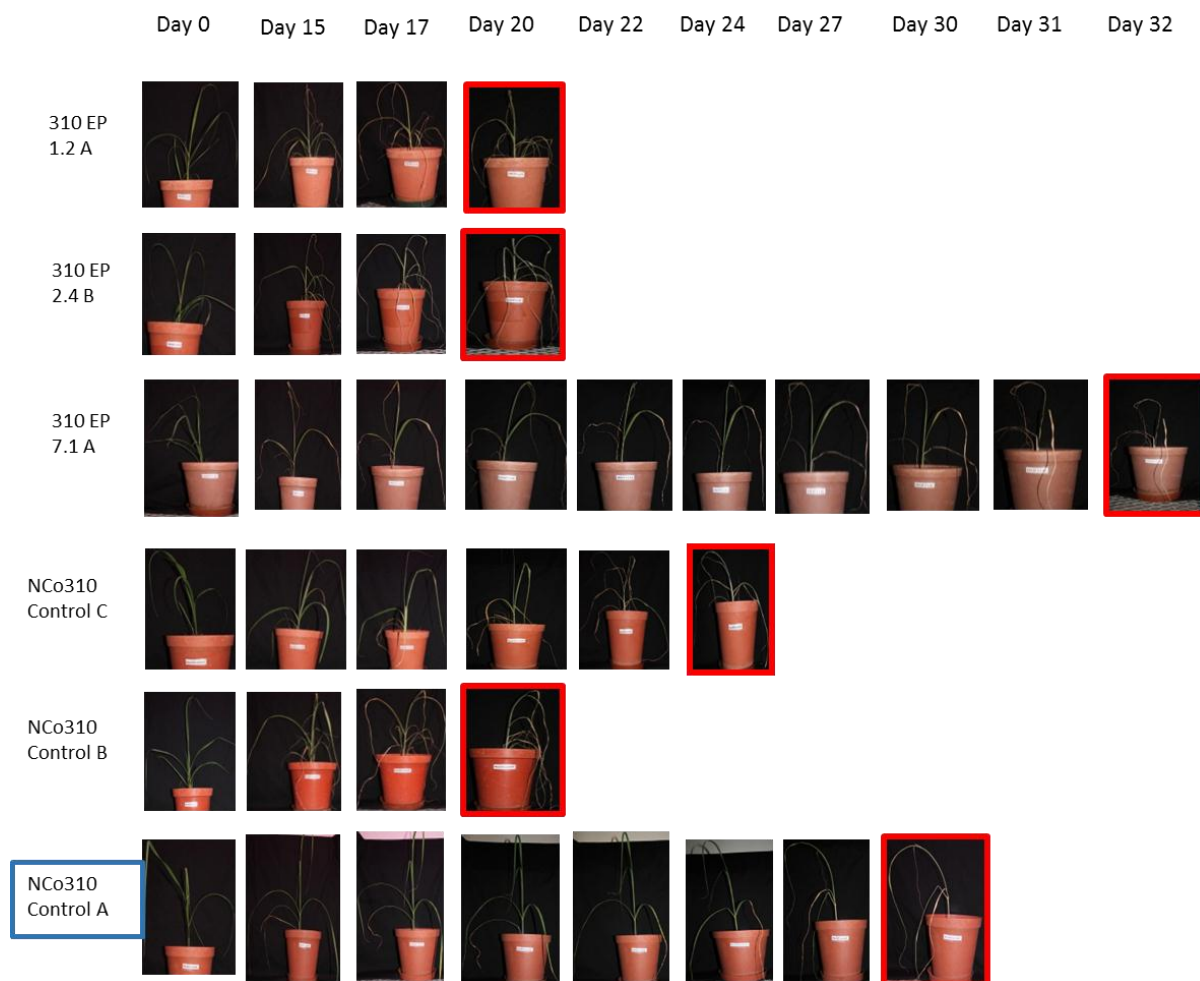
Figure 2.24: EMS treated NCo310 drought pot trial 2. Phenotypic monitoring of the second preliminary drought pot trial over time. Pictures outlined with red represent the day that the respective plant died. The plant highlighted in blue represents the NCo310 control plant for which the soil moisture content was not consistent with the rest of the pots in the trial.

2.2.4.3 Follow-up drought pot trials

Selected plantlets that outlived the control plant sets in the preliminary drought trial 1 described in section *2.3.4.2*, (plants 310EP 4.4A, 4.5A and 1.1C) as well as the selected 310EP 4.1A plant which showed signs of recovery after apparent death were subjected to a further drought trial. For this, plants were subjected to water deficit for a period of 36 days. Daily soil moisture content and phenotypic evidence of plant survival or death over drought duration were recorded for each planted pot. In this study, we considered a selected line to be potentially drought tolerant when it outlived two of the three biological replicates from the NCo310 control plants.

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A uniform decline in soil moisture was observed in all lines, with the exception of clone 310EP 4.4B and 310EP 4.5B (Figure 2.25). Data represented in Table 2.2 showed that plant death during water deficit conditions were seen on average 27 days after water was withheld for NCo310 control plants, . Clones 310EP 4.1 died on similar days as that of the controls. Mutant lines 1.1, 4.4 and 4.5, died on average on days 30, 31 and 31, respectively, outliving all control sets with a minimum of three days (Table 2.2).

Three days after plants were presumed dead, each plant was re-watered to evaluate any recovery. As in the preliminary drought trial 1, only 310EP 4.1 recovered and formed new shoots, although only one clone showed this phenotype (Table 2.2). However, this experiment will have to be repeated in the future with proper biological repeats before conclusions are made.
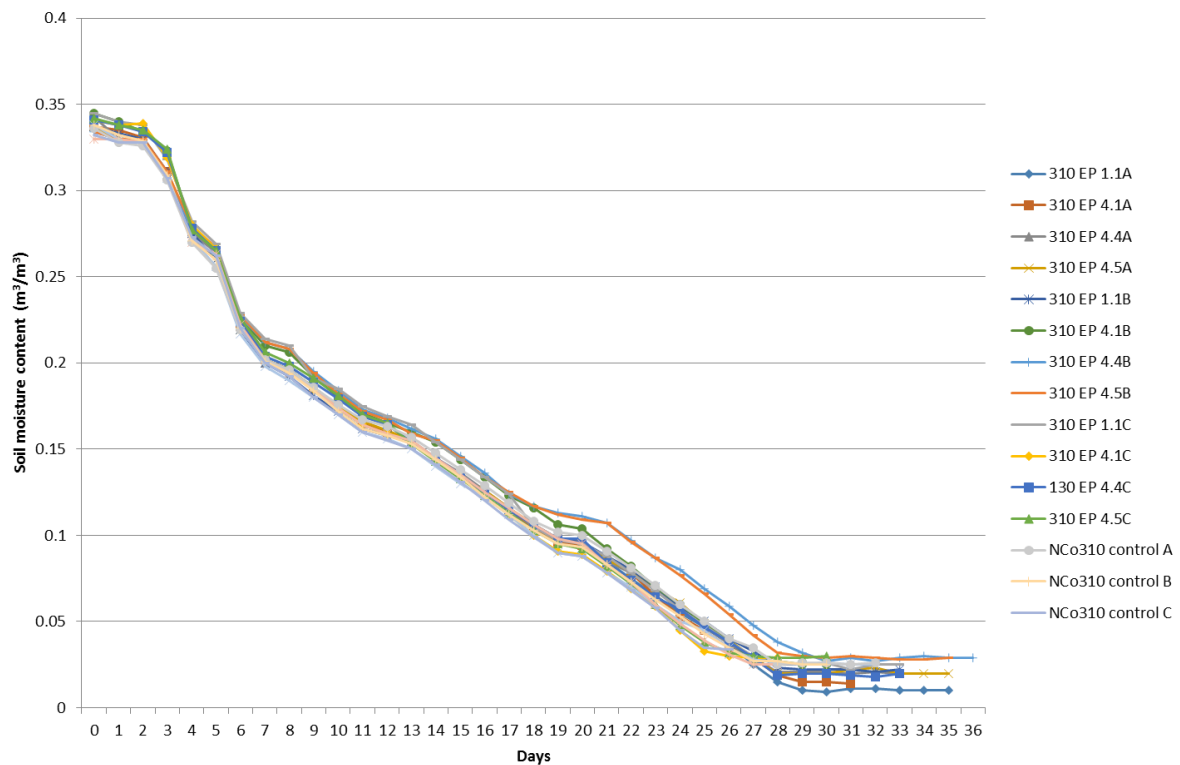
Figure 2.25: Drought pot trial of the EMS treated NCo310 clones. The graph illustrating a uniform decrease in soil moisture content in each pot over time with the exception of clone of 310EP 4.4 B and 4.5B which were slightly higher. NCo310 control represents the most drought tolerant cultivar that provides a threshold for potential drought tolerance in experimental lines.

**Table 2.2:** A drought trial of multiple clones of each EMS treated NCo310 line selected from preliminary drought pot trial 1. For each pot the day of presumed plant death are indicated with an X. R represents the day of plant recovery following re-watering. Mutant clone 4.1C was the only plant that when re-watered recovered after water deficit.

Plant Line	Drought trial time point (days after drought initiation)															R
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	42
310EP 1.1 A											X					
B										X						
C										X						
310EP 4.1 A							X									
B							X									
C	X															X
310EP 4.4 A										X						
B															X	
C											X					
310EP 4.5 A											X					
B											X					
C					X											
NCo310 A							X									
B							X									
C			X													

2.4. Discussion

2.4.1. *In vitro* cultures

In vitro sugarcane regeneration was initiated in Hawaii, in 1961 (Liu, 1984; Chengalrayan *et al.*, 2005), since then, sugarcane cultivars have been successfully regenerated *in vitro*. These cultivars include N12, N16, N21 and NCo310, among others that originate from South Africa (van der Vyver, 2010; http://www.sasa.org.za). In this study we investigated the *in vitro* regeneration abilities of three *Saccharum* hybrid cultivars of Southern African origin displaying different levels of drought tolerance. The tested cultivars showed variation in their ability to form embryogenic callus and multiple shoots via somatic embryogenesis. The sugarcane cultivar that showed the highest *in vitro* coefficient was NCo376, while N40 displayed the lowest coefficient. Similar results and variation between sugarcane cultivars' *in vitro* regeneration was also found in previous studies as well as in many other plant

species (Gandonou *et al.*, 2005; van der Vyver, 2010; Phillips and Collins, 1979; Plana *et al.*, 2006; Shan *et al.*, 2000).

What was clear from this study was that the 2,4-D concentrations in the culture media played an important role in the *in vitro* performance of the cultivars. Each cultivar reacted differently, in terms of callus induction, towards the different concentrations of auxin (2,4-D) used in the growth media. Compared to other synthetic auxins such as naphthaleneacetic acid (NAA), 2,4-D has been shown to induce callus best in the majority of plant species (Phillips and Collins, 1979; Shan *et al.*, 2000; Maddock *et al.*, 1982), this rendered 2,4-D the primary choice of auxin used in plant cell cultures. Past literature also mainly used 2,4-D as primary auxin for callus induction in sugarcane (Behera and Sahoo, 2009; Koch *et al.*, 2012). Sugarcane shoots and roots induction have been shown to be profuse in MS media supplemented with plant hormones such as 6-benzylaminopurine (BAP) and NAA (Behera and Sahoo, 2009). However, in this study we were able to induce shoots and roots on either MS or ½ MS media without the addition of any hormones for all cultivars.

2.4.2. Chemical mutagenesis

In the past chemical mutagens such as sodium azide, methyl methanesufonate (MMS) and ethyl methanesulfonate (EMS) have been used to induce point mutations in plant genomes, resulting in either gain-of-function, such as herbicide resistance, or loss-of-function, such as reduction of lignin in plant cell walls by targeting genes such as caffeic acid 3-*O*-methyltransferase (COMT). a series of change-of-function mutations. However, among these chemical mutagens, EMS has the highest mutation rate and has been shown to produce genome-wide non-lethal mutations in numerous plant species with easy application (Bhat *et al.*, 2007; Auerbach and Robson, 1946; Comai and Henikoff, 2006; Till *et al.*, 2003; Parry *et al.*, 2009; Talebi *et al.*, 2012; Hohmann *et al.*, 2005; Serrat *et al.*, 2014; Koch *et al.*, 2012; Jander *et al.*, 2003).

When EMS was used in this study to induce mutations in the *Saccharum* hybrid species cultivar NCo310, no clear conclusion on the effect of the different EMS concentrations could be made based on visual assessment of cultured callus and regenerating shoots. This included the observation of genetic off types displayed as chlorophyll deficiencies and growth reduction in callus and shoots. Ideal mutagen concentrations can vary according to the type of mutagen used and the plant species, cultivar and tissue type which are exposed to the mutagen during the experiment. Kovalchuk *et al.* (2000) for example mutated *Arabidopsis thaliana* callus tissue and induced mutations at a rate varying between 10^{-7} to 10^{-8} chances of mutation occurring per gene copy per generation using 50 µM methyl

methanesulfonate (MMS), while EMS has been used in the past to mutate callus cells from rice and sugarcane at concentrations varying from 8 to 16 mM (Koch *et al.*, 2012; Serrat *et al.*, 2014). An important outcome from EMS application in this study was that 16 mM EMS resulted in a number of shoots that were considered drought tolerance.

Also important is the duration the plant tissue is exposed to the mutagen. Exposure should generate useful amounts of mutations without killing plants or hindering embryogenicity (Koch *et al.*, 2012). In plant species, such as wheat, soybean, sugar beet and sweet potato optimal EMS concentrations range from 8 – 40 mM with 1 – 3 hours exposure time (Sung, 1976; Masrizal *et al.*, 1991; He *et al.*, 2009; Hofmann *et al.*, 2004; Luan *et al.*, 2007; Kenganal *et al.*, 2008; Omar and Novak, 1990). Furthermore, Kenganal *et al.* (2008) determined 40 mM EMS for 2.5 hours exposure of sugarcane callus was sufficient for producing salt-tolerant plants. In a study conducted by Koch *et al.* (2012), callus exposed to 16 mM EMS for four hours was optimal for producing desired mutagenic sugarcane clones.

Studies have shown that both seeds and callus cells are able to be mutated using EMS (Koch *et al.*, 2012; Kumar *et al.*, 2015). However, according to Kilbey and Hunter (1983), EMS is most effective when applied to dividing cells, such as proliferating callus, as cells are engaged in DNA replication and the probability of high mutation induction is greatest. Based on this, callus was the choice of explant material for mutation induction for this study. The fact that sugarcane does not flower easily, would also limit the availability of seeds which made callus cells the only viable option for our mutation experiments.

2.4.3. *In vitro* osmotic treatment

To produce drought tolerant plants *in vitro*, the optimal osmoticum concentration had to be used in order to mimic a drought environment. The ideal osmoticum and its optimal concentration would, without being taken up by callus cells, lead to significant cell necrosis and no signs of normal plant survival or regeneration.

In this study mannitol and PEG osmotica were tested. Mannitol treatment described by Errabii *et al.* (2007) using concentrations ranging from 100 to 300 mM showed significant cell stress in sugarcane. However, this was not observed in the current study, where no significant differences were found between control sugarcane calli and that exposed to these lower mannitol concentrations. As seen in Figure 2.10, when calli were exposed to higher mannitol concentrations of between 400 mM and 1 M, the 700 mM and 1 M concentrations significantly reduced callus growth and led to cell death. However, out of concern that these high mannitol concentrations might result in cell death due to toxicity induced by the plants'

natural metabolism of this sugar, and not because of osmotic pressure from the decreased water potential in the growth environment (Fritz and Ehwald, 2010; Lipavska and Vreugdenhil, 1996), the use of this osmoticum was excluded from further experimentation during this study.

When testing PEG, the initial treatment followed the method as described by Wani *et al*. (2010). Here, PEG (6000) concentrations of between 0.5 to 2% were used to evaluate water stress in rice (*Oryza sativa*) seeds selected for four weeks. This study validated the use of PEG as appropriate osmoticum *in vitro* and demonstrated cultivars that were relatively tolerant to drought stress. However, in this current study neither of these concentrations were high enough to result in sugarcane callus death suitable to select for osmotic tolerance. An experimental design described by Rao and Jabeen (2013) on sugarcane callus increased the PEG concentrations to between 10 to 30%. High PEG concentrations resulted in problems with growth media solidification. To overcome this, solid media, containing 30% PEG was prepared as described by Verslues and Bray (2004) using an infiltration overlay approach. Nevertheless, solid media containing 30% PEG did not result in callus necrosis which might be due to the infiltration protocol used to prepare the media ultimately lowering the final amount of osmoticum present within the growth medium (Figure 2.13 A and B). On the other hand, calli exposed to 30% PEG in liquid media seem to be too harsh resulting in the inability of cells to proliferate at all after the treatment (Figure 2.15 A and B). Therefore, after looking at cell responses to all PEG treatment concentrations, exposure to 20% PEG seemed to have the desired response of producing cell necrosis and limited shoot regeneration ability on both solid and liquid cultures. These results were similar to those described by Rao and Jabeen (2013), where between 20 and 30% PEG resulted in significant sugarcane callus size reduction and browning (necrosis).

In addition to the osmoticum concentrations, the selection duration on the osmoticum also plays a part in establishing a successful selection regime. When callus clumps were exposed to PEG for only a short duration, especially on solid media, cell clumps might shield cells against the effect of the PEG treatment, therefore resulting in indiscriminate cell proliferation and growth after selection. In liquid media however, small cell clumps are more evenly exposed to the selection agent which might result in reduced selection time periods needed to achieve the desired effect. In a study done by Ahmad *et al*. (2007), rice cultivars were selected *in vitro* for drought tolerance using PEG (10 – 29%) liquid media for a period of 15 days, which was sufficient to identify desired clones. However, this was not the case in the current study. Lengthy exposure times of up to 16 weeks, with a two week recovery phase in the middle, was necessary to induce necrosis in most of the exposed cells while still allowing some level of *in vitro* regeneration (Figures 2.11 A, 2.13 C and 2.15 C). To

conclude, in terms of determining the optimal parameters for sugarcane *in vitro* osmoticum tolerance, NCo310 callus responded optimally (least callus growth, regeneration and survival) when exposed 20% PEG on a eight week selection: two week recovery: eight week selection (eight weeks PEG selection in the dark: two weeks PEG recovery in the light without PEG exposure: eight weeks PEG selection in the light) time regime.

2.4.4. Drought tolerant clones

Mutation breeding is an alternative solution for creating crops with improved abiotic stress tolerance. It has been used very successfully to create a large number of crops with enhanced properties such as insect and herbicide resistance as well as tolerance to abiotic stresses such as cold, salt and drought, in a wide range of plant species that include banana, sweet potato and wheat (*Triticum aestivum*) (Reyes-Borja *et al.*, 2007; He *et al.*, 2009; Shu *et al.*, 2011; http://mvgs.iaea.org/). This strategy has been applied in the past only in a limited manner to sugarcane and can potentially be exploited more in this crop species.

In this study we were able to regenerate 19, 7 and 18 potentially osmoticum tolerant clones from the three sugarcane cultivars, NCo310, NCo376 and N19 respectively, using EMS mutagenesis and *in vitro* PEG selection. Of the 19 NCo310 clones that demonstrated potential osmoticum tolerance, five clones showed potential drought tolerance in preliminary greenhouse pot trials. During these trials, the soil moisture content (SMC) slowly decreased from fully saturated soil (average soil moisture content of 0.307 m^3/m^3) to dry soil (average soil moisture content of 0.016 m^3/m^3) over a period of 30-35 days. It was important to extent the water deficit period as long as possible to mimic natural field drought conditions as closely as possible during the experimental period. This is the opposite of studies where drought stress for short periods of times (hours) were considered as optimal stress regimes in rice and maize drought trials (Lu *et al.*, 2009 and 2011). According to Blum (2014), drought stress can be defined by three parameters, namely timing, severity and duration. By extending the drought period, mutant clones with realistic enhance tolerance traits will more likely be identified due to plants changing physiologically to accommodate the stress over an extended period of time. Therefore, this study extended the duration of a drought simulated environment over an extended time period. This was also in contrast with the methods chosen by, for example Rao and Jabeen (2013) where drought conditions were artificially extended by replacing the small amount of moisture lost each day by watering on alternating days for a period of 20 days. In such a scenario the plant undergo daily cycles of hydration and dehydration which is not ideal (Blum, 2014). Furthermore, plants were grown in pots

large enough to allow free root development and slow soil drying, and a mix soil medium that allowed uniform drying of pots was used.

According to Blum (2014), the developmental stage at which drought stress is induced in plants is of importance. For this study, the age of the plants used for drought trials was primarily chosen due to project time constraints. In this regard, water stress was induced on sugarcane plants that had been allowed to *ex vitro* acclimatise and grow for four weeks in the glasshouse. Ideally plants should be allowed to mature more in the future before exposure to drought stress conditions. Sugarcane plantlets should mature for approximately three months to be suitable for drought trials (SASRI communications). For sugarcane growth, phases most sensitive towards drought conditions are the germination and establishment, tillering and grand growth phases which on average, cover the growth period from 7-10 to 270 days during a sugarcane crop growth cycle of 12 months and should eventually be the focus period for a drought trial in the future.

It is furthermore difficult to find information regarding the status of sugarcane cultivars performance under drought conditions, and which can be considered as drought resistant or tolerance genotypes. Sugarcane is a C_4 grass which, during drought conditions, will most likely experience physiological changes such as extended roots, changes in leaf structure and development, as well as stomatal conductance (Smit and Singels, 2006; Carmo-Silva *et al.*, 2009). Nevertheless, the cultivars used in this study have not previously been formally graded based on their drought tolerance capabilities. The only information regarding this was communicated to us by the South African Sugar Research Institute (SASRI), and which is based on informal observations of the performance of these cultivars on commercial farms. No technical drought stress studies have been conducted as yet on these cultivars. They are described by SASRI as relative drought tolerant (NCo310) and drought susceptible (NCo376 and N19) cultivars (Inman-Bamber, 1982). However, NCo376 was reported to be more susceptible than N19 (http://www.sasa.org.za; McIntyre and Nuss, 1996). Furthermore, N19 is most susceptible to water stress when planted on shallow soils with more than 20% clay content (http://www.sasa.org.za; McIntyre and Nuss, 1996). The *in vitro* osmoticum selection regime preliminarily indicated that N19 is more drought resistant than NCo376, since the total number of N19 shoots surviving the *in vitro* osmotic selection were almost three times more than that of NCo376 (Figure 2.18 C). However, this will have to be confirmed in the future with at least greenhouse drought pot trials.

From the 19 identified first generation NCo310 mutant clones (M1 clones), only five outlived the NCo310 wild type controls in the preliminary drought pot trials. One clone, when re-watered three days after assumed plant death, displayed the ability to initiate re-growth after

the drought regime (Figure 2.22 and Table 2.2). This means that in this study, 33% of the initial *in vitro* osmotic tolerant NCo310 EMS treated clones that were identified *in vitro* developed into individuals with enhanced drought tolerance that could be confirmed during the *ex vitro* drought pot trials.

Possible reasons for the relative high percentage of clones that were initially identify with potentially osmotic tolerance during the *in vitro* selection but which did not display the same trend during the *ex vitro* drought pot trials, might be due to the chosen developmental stage of the plants in the pot trials or the *in vitro* PEG selection design that was not stringent enough. The duration of PEG exposure might have to be further extended to remove more false positives that manage to recover during the two week recovery phase *in vitro*. The option of exposing single cells to PEG media through liquid cell suspension cultures, instead of exposing small callus clumps to solid media might increase exposure of PEG to individual cells and reduce chances of recovery and survival of false positive clones. Lastly, although callus cells are able to regenerate into plants, the two cell structures are very different in terms of physiological structure, and therefore will not respond exactly the same way to abiotic stresses. Callus cells are grown under controlled environments with stimuli that mimic environmental abiotic stresses, they are therefore useful for demonstrating how plants will respond to environmental abiotic stresses. The use of plantlets *in vitro*, instead of callus cells to study abiotic stresses, though time consuming, might be useful in reducing the number of false positive clones.

In the future, preliminary drought trials will have to be conducted and repeated with identified M1 clones to gather data such as relative leaf water content in the plants, stomatal conductance, photosynthesis rate, root architecture and water potential regarding these enhanced drought tolerant phenotypes. Also, these plants will have to be assessed under non-stress environments to confirm normal growth phenotypes, structure, susceptibility to other pests & diseases and sucrose levels. In the long term, following drought pot trials, field trials will be executed to confirm drought tolerance of selected plants and further validate the experimental design in a natural environment.

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Chapter 3

Genetic manipulation of sugarcane by overexpression of a glucoronokinase gene

3.1 Introduction

The fluctuating cost of petroleum alongside its association with adverse influences on the environment, have propelled interest in searching for energy alternatives without compromising food security (Babcock, 2011). Biofuels are, in this regard, a potentially attractive solution to ameliorate these problems.

Second-generation biofuels are produced from plant lignocellulosic biomass, which in this context, are agricultural by-products of plants primarily used as food sources (Naik *et al*., 2010). Their production ideally depends on the breakdown of bonds between carbon atoms within the plant cell wall lignocellulosic components. Sugarcane produces high yields of sucrose and cell wall lignocellulosic biomass and can therefore serve as both a significant food and biofuel source.

Sugarcane cell wall lignocellulosic biomass remains after sucrose extraction and is referred to as bagasse. This is composed of 45-50% cellulose, 25-35% hemicellulose and 25-35% lignin and ash with traces of pectic polymers (Reshamwala *et al*., 1995; Cheung and Anderson, 1997; Boopathy, 1998; Dewes and Hünsche, 1998) (Figure 3.1). Bagasse is primarily used as a second-generation biofuel source.   In terms of the biological structure and function of the plant cell wall, the cellulose, hemicellulose and pectic polymers assist lignin in provision of cell wall structure, mechanical support, resistance to abiotic and biotic stresses, pests and disease, as well as nutrient and water transport throughout the plant (Chabannes *et al*., 2001; Jones *et al*., 2001; Sarkanen and Ludwig 1971).

Lignin is a complex phenolic biopolymer synthesised by the radical coupling of three hydroxyphenylpropanoid monomers (monolignols) through a series of oxidations catalysed by extracellular enzymes, peroxidases and laccases (Morreel *et al*., 2004; Vanholme *et al*., 2010). Considering the above-mentioned functions of lignin in wooded plants particularly, it is of particular importance because it also provides plant rigidity and therefore its degradation cannot be an effortless task.

Cellulose needed to produce biofuels is trapped inside pectic polymers, lignocellulosic polymers and lignin via ester and ether bond cross-linkages. This complex structure has, as

mentioned before, evolved to resist degradation to confer hydrolytic stability and structural robustness to plant cell walls (Chen and Dixon, 2007). To extract cellulose for bioethanol production, the cross-linkages between lignin, hemicellulose and pectin polymers first need to be broken down to allow hydrolysis of the exposed cellulose to simple monosaccharides which will be fermented to bioethanol. This process in bioethanol production is the most important and rate limiting as it requires expensive equipment as well as high energy inputs (Chen and Dixon, 2007).
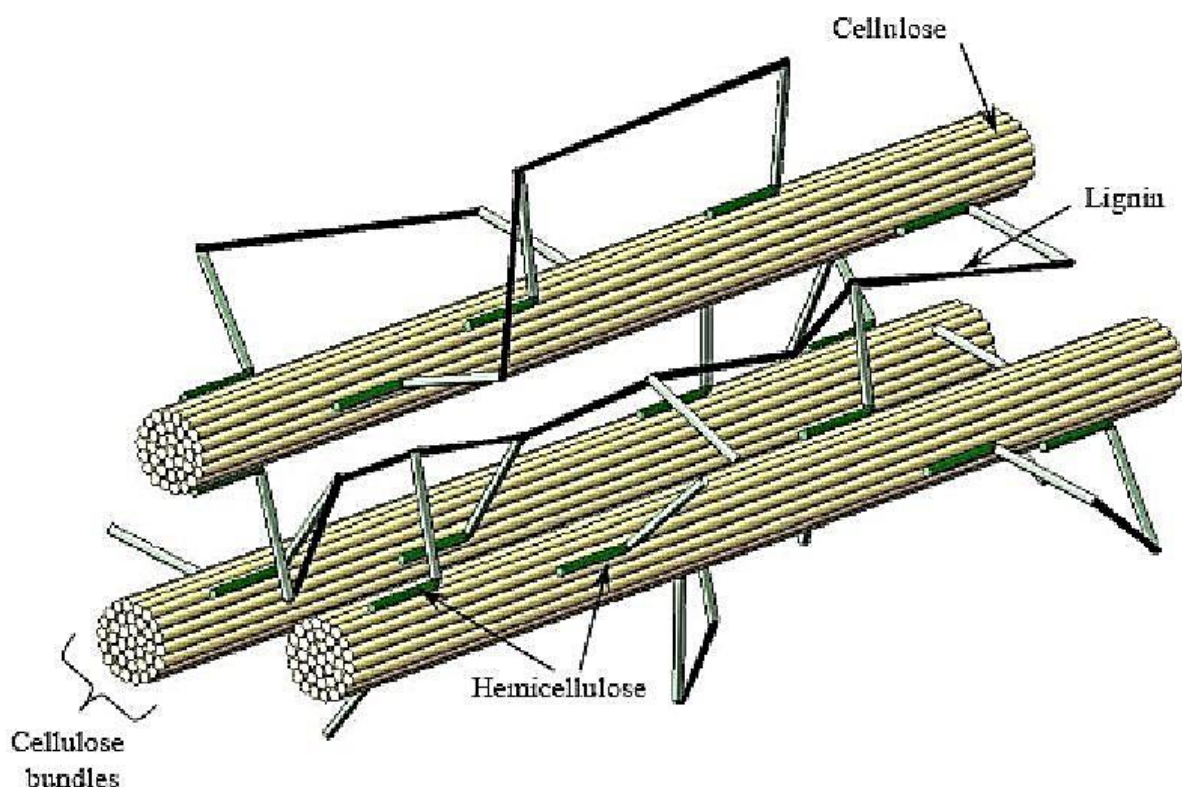
Figure 3.1: Biological structure of lignocellulosic biomass in plant cell walls (www.intechopen.com).

Plant cell wall components, lignocellulosic biomass (hemicellulose and pectin polymers) as well as sugars uridine 5'-diphosphate (UDP)-xylose, UDP-arabinose, UDP-apiose and UDP-galacturonic acid) are produced from UDP-D-glucuronic acid (UDP-Glc A), which is synthesised by two pathways. Firstly, the sugar nucleotide oxidation pathway (SNOP) uses UDP-glucose to form UDP-Glc A catalysed by UDP-glucose dehydrogenase (UGD). Secondly, the *myo*-inositol oxidation pathway (MIOP) uses inositol to form UDP-Glc A by the enzymes, inositol oxygenase, glucuronokinase, and UDP-sugar pyrophosphorylase (USP) (Loewus and Murthy, 2000; Loewus *et al.*, 1962; Seifert, 2004) (Figure 3.2). It has not yet been clarified which pathway is most important for the production of UDP-Glc A for cell wall

synthesis. The MIOP has been shown to be present in a variety of plant tissues ranging from shoot and root tips, leaves to pollen (Dickinson, 1982). The counterbalance of inositol and glucose in different developmental stages determines which pathway will form cell wall polysaccharides. For example, Loewus and Loewus (1980) determined that, in germinating *Lilium longiflorum* (Easter lily) pollen, the MIOP only plays a major role in cell wall polysaccharide formation when inositol levels are high or glucose levels are low. Whereas Seitz *et al.* (2000) showed that the MIOP was dominant in young *Arabidopsis thaliana* seedlings, a developmental stage where expression of UGD is absent. That study also demonstrated that UGD expression is primarily confined to root tissues and showed an increase of UGD activity in leaves and hypocotyl of older seedlings, which remained uniform throughout the vegetative phase of the plant, but declined during senescence. Since UDP-Glc A is required in all plant tissues at all developmental stages, Seitz *et al.* (2000) radiolabelled young seedlings with *myo*-inositol to determine whether the MIOP contributes toward UDP-Glc A synthesis in *Arabidopsis*. The results showed incorporation of radioactive label preferentially in cotyledons and the hypocotyl, tissues where the UGD gene was weakly expressed (Reiter and Vanzin, 2001).
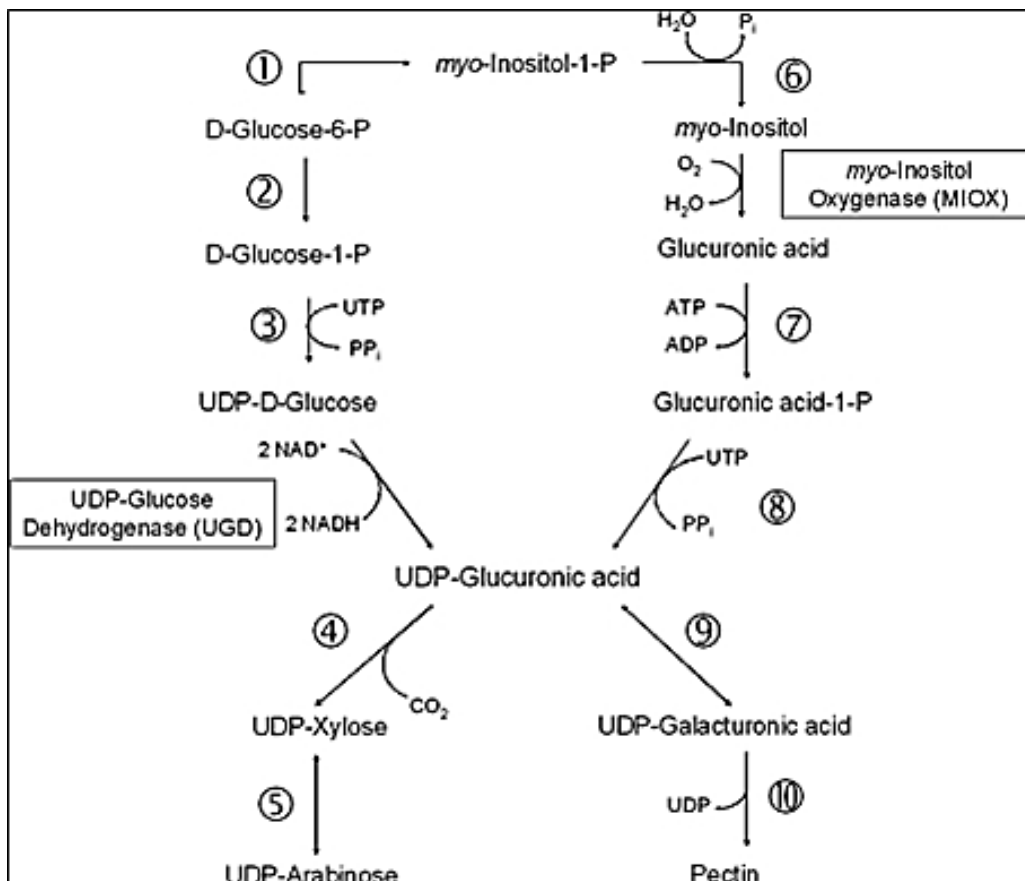
Figure 3.2: Biosynthesis of UDP-glucuronic acid and related cell-wall precursors. ① Inositol-1-P-synthase; ② Phosphoglucomutase; ③ UDP-glucose pyrophosphorylase; ④ UDP-glucuronic acid decarboxylase; ⑤ UDP-arabinose 4-epimerase; ⑥ Inositolphosphate-phosphatase; ⑦ Glucuronokinase; ⑧ UDP-sugar pyrophosphorylase; ⑨ UDP-glucuronic acid 4-epimerase; ⑩ α-Galacturonosyl transferase (Endres and Tenhaken, 2011). Reaction ① - ⑤ confer to the sugar nucleotide oxidation pathway (SNOP); Reaction ⑥ - ⑩ confer to the *myo*-inositol oxidation pathway (MIOP).

A study conducted by Bekker (2007) demonstrated that UGD repressed sugarcane lines resulted in an increase in both sucrose and cell wall cellulose content, the latter being a characteristic that would render the plants improved for use in second-generation biofuel production. Furthermore, uronic acids such as glucuronic and galacturonic acids, as well as cell wall pentose sugars were also increased. To understand the increase in uronic acids and cell wall pentose sugars, Bekker (2007) measured the activity and expression of the first enzyme of the MIOP, *myo*-inositol oxygenase (MIOX) in young sugarcane leaves. Both activity and expression of the MIOX were upregulated in transgenic lines when compared to of the wildtype. Bekker (2007) hypothesised that these results indicate that when the SNOP is repressed, the MIOP compensates for the decreased synthesis of UDP-Glc A (Labate *et al.*, 2008; Bekker, 2007). The second enzyme of the MIOP pathway, glucuronokinase, might also play a role in the assumed upregulation of this pathway in UGD repressed lines, but this is still unknown and need to be investigated. To try and understand the reasons for the increased sucrose, Bekker (2007) measured the enzyme activity of sucrose synthase (SuSy) and sucrose phosphate synthase (SPS). Although SuSy activity normally acts in the breakdown direction *in vivo,* the enzyme did not show any changes in either sucrose breakdown or synthesis in young and mature sugarcane internodes in this study. However, both enzymes showed significant increase in activity in the culm tissue of UGD repressed lines, which suggested that the sucrose synthesis capacity of these transgenic plants as well as the specific SPS activity or activation were in some way enhanced but without any specifications as to why or how. This study therefore aimed to build on the study of Bekker (2007) by determining if there are changes in sucrose content in glucuronokinase overexpressing sugarcane. Since cellulose and hemicellulose form the majority of lignocellulosic biomass increasing these components in crop cell walls might result in cell walls containing reduced lignin and, therefore, crops with increased cell wall components that can be used for biofuel production.

Glucuronokinase catalyses the reaction converting D-glucuronic acid and adenosine triphosphate (ATP) to D-glucuronic acid-1-phosphate and adenosine diphosphate (ADP).

This reaction is part of the *myo*-inositol oxidation pathway (MIOP), during which inositol is oxidised to glucuronic acid, a precursor of both cell wall sugars and lignocellulosic biomass (Loewus and Dickinson, 1982). This enzyme was first isolated from *Phaseolus aureus* (mung bean) seedlings by Neufeld *et al.* (1959), however it is still relatively unstudied. Leibowitz *et al.* (1977) partially purified this kinase from *Lilium longiflorum* (Easter lily). However, no full length gene could be identified in any organism for this enzyme until Pieslinger *et al.* (2010) cloned and characterised the enzyme from *L. longiflorum* by transformation into *Arabidopsis thaliana.* They concluded that the enzyme is a novel sugar-1-kinase belonging to the family of GHMP (galacto, homoserine, mevalonate and phosphomevalonate) kinases. Members of this enzyme family are ATP-dependent enzymes involved both in the biosynthesis of amino acids and carbohydrate metabolism (Pieslinger *et al.*, 2010).

Nucleotide sugars are activated forms of monosaccharides that primarily act as glycosyl donors in reactions catalysed by glycosyltransferases to produce cell wall biopolymers, proteoglycans, glycolipids, glycosylated secondary metabolites and glycoproteins (Reiter and Vanzin, 2001; Bar-Peled and O'Neil, 2011). Two major pathways for direct synthesis of nucleotide sugars from phosphorylated monosaccharides are the production of UDP-D-glucose from uridine 5'-triphosphate (UTP) and glucose-1-phosphate, and the synthesis of guanosine 5'-diphosphate (GDP)-D-mannose from guanosine 5'-triphosphate (GTP) and mannose-1-phosphate. An alternative pathway for synthesis of UDP-D-glucose is catalysed by sucrose synthase which converts UDP and sucrose to UDP-D-glucose and fructose. Other nucleotide sugars are synthesised by the sequential action of monosaccharide kinases and nucleotide 5'-diphospho (NDP)-sugar phosphorylases through salvage and NDP-sugar interconversion pathways (Reiter and Vanzin, 2001). Nucleotide sugars may also act as signalling molecules interacting with the hormonal signalling network regulating the plant immune system (Morkunas and Ratajczak, 2014). Infection by a fungal pathogen can lead to increased lignification, and increased nucleotide sugars in cell walls under these circumstance (Morkunas and Ratajczak, 2014). Hexokinases are enzymes that phosphorylate six carbon sugars to hexose phosphate. In most organisms, glucose is the most important substrate, while glucose-6–phosphate is the most important product. In addition, they have been intensively studied in sugar signalling as glucose sensors in plants (Morkunas and Ratajczak, 2014; Rolland *et al.*, 2006). Because Bekker (2007) hypothesised that when the SNOP was compromised, the MIOP assumed the function of producing uronic acids such as glucuronic and galacturonic acids, of which glucuronic acid is a precursor for cell wall biopolymers, it may be assumed or hypothesised that the MIOP has sugar signalling abilities. Although glucuronokinase has not been shown to be involved in

sugar signalling, it is however, like hexokinase an enzyme involved in production of nucleotide sugars. Glucuronokinase therefore has the possibility of being involved in sugar signalling in the MIOP.

Based on the assumed involvement of glucuronokinase in plant cell walls, this study aims to increase sugar (lignocellulosic) and possibly sucrose abundance in sugarcane lines overexpressing the glucuronokinase gene isolated from *Arabidopsis thaliana*.

3.2 Materials and Methods

3.2.1. Chemicals

Chemicals were purchased from Sigma Aldrich (St. Louis, USA) and Merck (Darmstadt, Germany), while genomic DNA and RNA extraction kits, plasmid DNA isolation kits as well as cDNA synthesis kits used were obtained from Qiagen (Dusseldorf, Germany), Promega (Madison, USA) and Thermo Scientific (Waltham, USA), unless otherwise stated.

3.2.2. Plasmid DNA isolation

Escherichia coli bacterial cells were transformed with the *Arabidopsis thaliana* glucuronokinase gene (At3g01640) that had been ligated in sense orientation into the pUBI 510 plant expression vector driven by the constitutive maize ubiquitin promoter and the CamV terminator. The pUBI 510::*Gluc* construct was provided by the Institute for Plant Biotechnology (Stellenbosch University, Stellenbosch, South Africa).

3.2.2.1 E. coli growth conditions

Transformed *E. coli* cells containing pUBI 510::*Gluc* were grown on solid Luria Bertani (LB) media containing tryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L. For solid LB media, 15 g/L agar was added. Media was autoclaved for 15 min at 121°C, prior to use. Selection of bacterial cells was induced by the addition of filter sterilized 100 µg/mL ampicillin to the media under sterile conditions. Under a laminar flow cabinet, 25 mL of LB media was poured per petri dish, once set, transformed *E. coli* cells were streaked out on the media and incubated overnight at 37°C.

Subsequent to the 37$^{°}$C overnight incubation, a 250 mL Erlenmeyer flask was filled with 150 mL liquid LB media containing 100 µg/mL ampicillin. A single colony was inoculated into the flask, which was subsequently incubated on a shaker overnight at 37°C.

3.2.2.2 Isolating plasmid DNA

Following growth of transformed *E. coli*, plasmid DNA (pUBI 510::*Gluc*) was isolated, as per instructed by the manufacturer, using the GeneJet Plasmid Maxi prep Kit (Thermo Scientific, Waltham, USA).

3.2.3. Genetic transformation of sugarcane callus

3.2.3.1 Callus induction

For *in vitro* sugarcane callus induction, *Saccharum* species hybrid cultivar NCo310 immature inner leaves from the apical segment of sugarcane stalks were used as explant material. The basal part of the inner leaf roll was aseptically isolated from the explant, sliced into approximately 3 – 5 mm thick leaf roll discs and placed on MS_3 media (pH 6.0) containing 4.4 g/L MS with vitamins (Murashige and Skoog, 1962), 20 g/L sucrose, 0.5 g/L casein, 3 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and 2.2 g/L gelrite. The media was autoclaved for 15 min at 121°C. Leaf discs were kept in the dark at 26°C for six to eight weeks and sub-cultured fortnightly for the duration of the callus induction phase. After approximately five to six weeks of induction, as callus began to develop from the leaf roll discs, it was removed aseptically from the discs and placed directly onto the media for additional callus growth.

3.2.3.2 Microprojectile bombardment of sugarcane

Sugarcane callus cells were transformed using a biolistic particle approach described by Bower and Birch (1992), with technical modifications introduced by the Institute for Plant Biotechnology. Four hours prior to transformation, induced NCo310 callus was placed on MS_3 media infused with 0.2 M of sorbitol and mannitol to create negative osmotic pressure.

For genetic transformation, 5mg of sterilised tungsten particles (M10, Bio-Rad, Hercules, USA) dissolved in 50 µl water were mixed with 10 µl of previously isolated plasmid DNA (1 µg/µL), 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine. The precipitate was allowed to settle on ice and 100 µl supernatant was discarded prior to bombardment. A locally constructed inflow gun was used for transformation, where 5 µl of the prepared particle solution was placed onto the centre of a 1 mm^2 metal grid. Sugarcane callus was co-bombarded with the pEmuKN selection vector (5 µg DNA of each vector), containing the neomycin phosphotransferase (*npt*II) gene, which allows selection of transformed callus on geneticin.

Callus induced on osmotic MS$_3$ media was placed 165 mm below the particle solution source and a pressure of 80 kPa was evacuated in the chamber prior to discharging of the particle solution. Following transformation, callus was re-placed on osmotic pressure MS$_3$ media for four hours before being transferred to basic MS$_3$ media for three days prior to selection. Bombarded callus was subsequently placed on MS$_3$ selection media containing 45 mg/L geneticin for eight to ten weeks in the dark at 26°C. Callus was sub-cultured every two weeks onto fresh media. Callus surviving the selection phase was transferred to MS media (MS$_3$ media without 2,4-D) and placed under cool fluorescent light (Osram; 36W/640) with intensity of 50 μmol photons/m^2/sec at 26°C with a 16/8 hour light/dark regime, for eight to ten weeks of shoot regeneration and plantlet formation. Plantlets with developed roots were hardened-off in 20 cm pots containing 2 parts potting soil, 1 part vermiculite and 1 part sand.

3.2.4. Molecular analyses of putatively transformed sugarcane

3.2.4.1 Primer design and synthesis

All primers were designed using the Primer 3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) online program and were synthesised at Inqaba Biotech (Pretoria, South Africa).

3.2.4.2 Polymerase chain reaction

GoTaq® DNA polymerase (Promega, Madison, USA) was used for all PCR reactions. Individual 50 µl PCR master mix reactions were assembled as follows: 10 µl of 5x green reaction buffer containing 7.5 mM MgCl$_2$, 1 µl of dNTP mix containing 10 mM of each dNTP, 10 µM each of the forward and reverse primers, 200 – 300 ng total DNA template, 1.25 U GoTaq® DNA polymerase and nuclease-free water to a total 50 µl reaction volume.

Thermal cycling conditions for GoTaq® DNA Polymerase-mediated PCR amplification were as follows: initial denaturation at 95°C for 2 min, 25-35 repeated cycles of denaturation at 95°C for 60 sec, primer annealing temperature at 42–65°C for 30 sec, extension at 72°C for 60 sec per 1 kb amplicon, and a final extension at 72°C for 5 min. Amplified DNA was stored at 4°C until required for use. The annealing temperatures differ based on the primer set used.

PCR products were separated, by electrophoresis, on a 1.5% (w/v) agarose gel containing 0.5% (v/v) Pronosafe (Separations, Johannesburg, South Africa) for amplicon visualisation. TBE buffer [5.4 g/L Tris base, 2.75 g/L boric acid and 0.465 g/L ethylenediaminetetraacetic acid (EDTA), pH 8.0] was used for making and running the electrophoresis gels. DNA bands

were separated at 100 mV for one hour with a lambda *PstI* marker. Separated DNA fragments were subsequently viewed under UV light.

3.2.4.3 Transgene confirmation

Putatively transformed plants were analysed for conformation of gene insertion using gene specific primers (Gluc Exp Forward: 5'- CGACCATCTCCTGAATCGTT -3' and Gluc Exp Reverse: 5'- CCTAAGCATTCATCCCCAAA -3'). Confirmation of gene insert was also performed using a combination of the Gluc Exp Forward and the plant vector's CamV reverse (5'- AGGGTTTCTTATATGCTCAAC -3') primer pair. Genomic DNA was extracted from plant leaves using the GeneJet Genomic DNA Extraction kit (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions.

3.2.4.4 Expression analysis

RNA was extracted from flash frozen leaves of transformed plantlets using the RNeasy Mini kit (Qiagen, Dusseldorf, Germany). For subsequent experiments, cDNA was further synthesised from 1 µg DNase I treated RNA using the RevertAid™ H First-strand synthesis cDNA kit as instructed by the manufacturer (Thermo Scientific, Waltham, USA).

SqRT-PCR was used to assess mRNA accumulation. The *ACTIN 2* gene was used as a housekeeping gene with the following primer pair, forward: 5'- TCACACTTTCTACAATGAGCT -3'; reverse: 5'- GATATCCACATCACACTTCAT -3'. Gene specific primers, (Gluc Exp Forward: 5'- CGACCATCTCCTGAATCGTT -3' and Gluc Exp Reverse: 5'- CCTAAGCATTCATCCCCAAA -3') were used to determine the level of transgene expression. PCR was performed and products were visualised as described in section *3.2.4.2*.

3.2.4.5 Plant growth conditions

Glucuronokinase transgenic and control NCo310 plants were planted at the end of 2014 in a growth tunnel at the Stellenbosch Welgevallen experimental farm under natural light and temperature conditions.

3.2.5. Sugar content analyses

3.2.5.1 Sugar extractions

Leaf material was harvested and ground into a fine powder using a mortar and pestle in liquid nitrogen. Sugars (glucose, fructose and sucrose) were extracted using an ethanol extraction method. For this, 100 mg ground tissue was added to 1150 µl of 80% (v/v)

ethanol and the mixture was incubated on a shaker for 30 min at 95°C before being centrifuged at 8000 g for 10 min. The resulting supernatant (S1) was collected. The pellet was subjected to a second and third round of extraction, using 700 µl of 80% (v/v) ethanol and 1150 µl of 50% (v/v) ethanol respectively, as described for the first extraction. All three supernatants were combined.

3.2.5.2 Sugar determination

To measure sugars, each microplate well contained 100 mM HEPES/KOH assay buffer (3 mM $MgCl_2$, pH 7.0), 100 mM ATP, 45 mM NADP, 60 U/mL G6PDH and the ethanolic extract (5 µl). Sugars were measured at OD_{340} with sucrose (2.5, 5, 7.5, 10 and 20 mM), glucose, fructose and NADPH (5, 10, 15, 20 and 40 mM) standards (Figure 3.3). When absorbance stabilised, 2000 U/mL HK (dissolved in 0.1 M assay buffer) was added to the mixture to measure glucose, when the reaction stabilised, 600 U/mL PGI (dissolved in 0.1 M assay buffer) was added to measure fructose and when the reaction stabilised, 500 mg invertase (dissolved in 0.1 M assay buffer) was added to measure fructose and glucose (sucrose). Change in OD_{340} was obtained to determine sugar concentrations expressed as µmol sugar x g^{-1} fresh weight (FW).
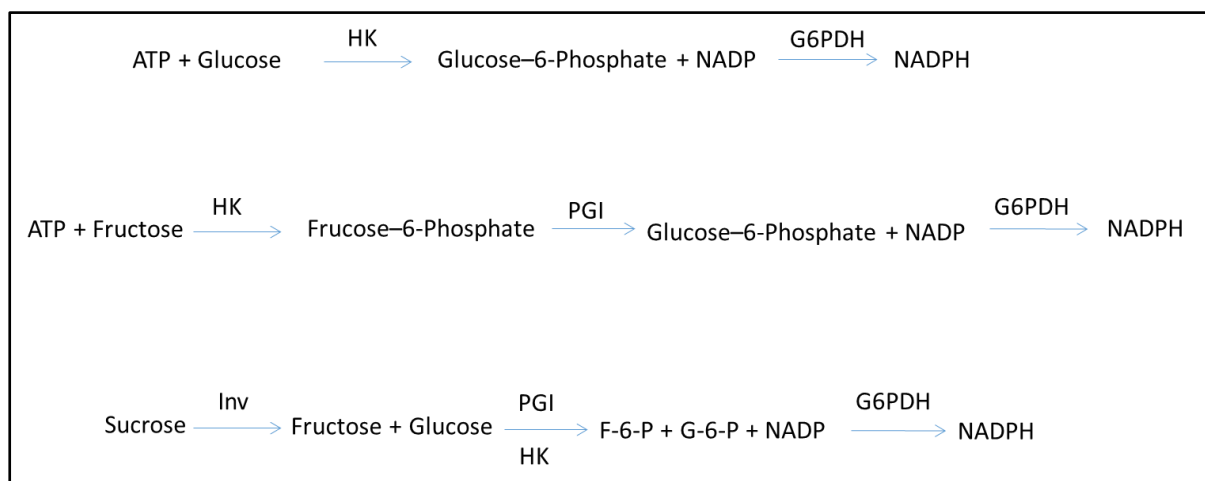

Figure 3.3: Biochemical pathway illustrating the principle on which the sucrose, fructose and glucose measurements are based. HK = hexokinase; PGI = phosphoglucose isomerase; Inv = invertase; G6PDH = glucose-6-phosphate dehydrogenase.

3.2.6. Enzyme activity analyses

3.2.6.1 Protein extractions

Transformed NCo310 sugarcane leaves were ground to fine powder using a mortar and pestle while suspended in liquid nitrogen. Under cold temperature conditions, 500 mg of ground tissue was homogenised with 2.5 mL protein extraction buffer [10 mM HEPES/KOH buffer (pH 7.4) containing 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 12% (v/v) glycerol]. The mixture was centrifuged (8000 g) at $4^{\circ}C$ for 30 min. The supernatant containing crude extract was collected and used for subsequent experiments.

3.2.6.2 Determination of protein concentration

Extracted crude protein concentrations were assayed using the Bradford (1976) method. For standard reference, 10 mg of bovine serum albumin (BSA) was diluted in 1 mL of sterile water and used in the dilution series of concentrations; 0, 15.75, 32.2, 62.5, 125, 250, 500, and 1000 µg/mL. Each microplate well contained a mixture of 10 µl of crude extract and 200 µl of Bradford reagent dye (Bio-rad, Hercules, USA). A spectrophotometer was used to obtain the OD_{595} reading of each sample.

A standard curve using absorption values of the BSA dilution series was constructed (protein concentration vs absorption) and was used to determine the protein concentration of extracts. Samples were diluted to achieve 5 µg of total protein prior to subsequent experiments.

3.2.6.3 Glucoronokinase activity assays

The assay buffer consisted of 80 mM MOPS/KOH (pH 7.5) buffer containing 10 mM $MgCl_2$, 25 mM KCl, 0.5 mM NADH, 1 mM phosphoenolpyruvic acid (PEP), 1 mM glucuronic acid, 1 U/mL pyruvate kinase (PK) and 1 U/mL lactate dehydrogenase (LDH).

Continuous assay

A total of 180 µl assay reagents including ~6 µg of protein crude extract, were added to each well to equilibrate the reaction at 340 nm for 15 min, whereby once stabilised, ATP was added to a final concentration of 1 mM to start the reaction which was run over a time period of 30 min (Pieslinger *et al.*, 2010).

Stopped assay

A stopped assay was performed to determine glucoronokinase activity by incubating 5.2 ug protein in 180 µL assay buffer (80 mM MOPS/KOH pH 7.5, 1 mM ATP and 1 mM glucuronic acid) for 30 min. The assay was halted by heating for 5 min at 95°C. ADP produced from

ATP present in the assay buffer was determined in an enzyme coupled assay using pyruvate kinase and lactate dehydrogenase at OD_{340} for 30 min as indicated by Figure 3.4.
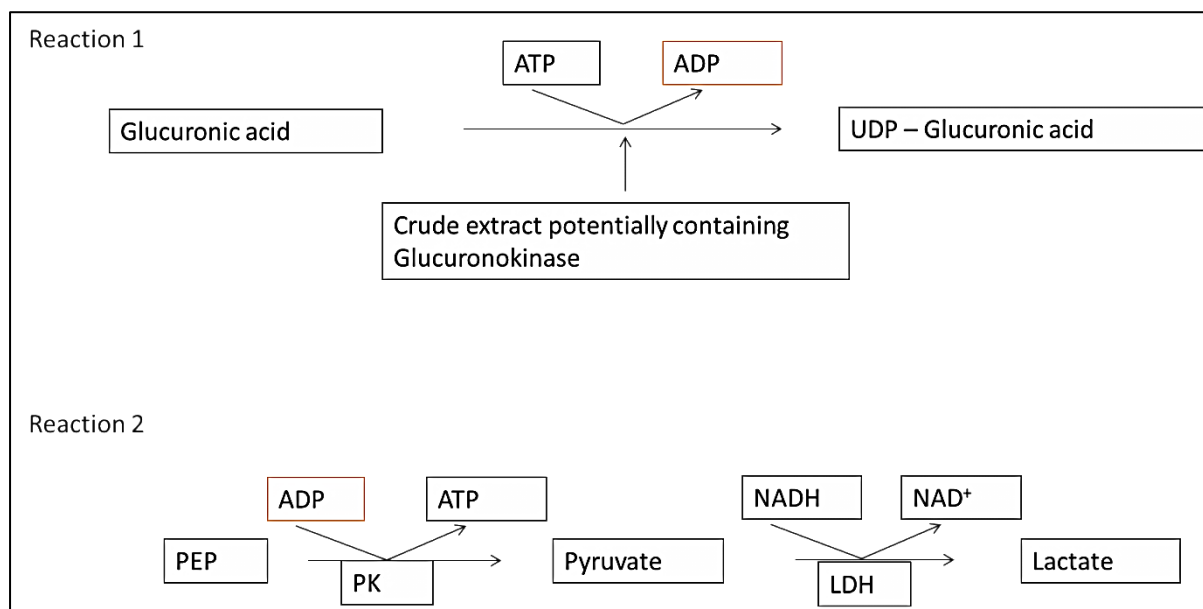

Figure 3.4: A schematic representation of the principle of a stopped enzymatic assay that was used to measure glucuronokinase enzymatic activity. ATP = Adenosine triphosphate; ADP = Adenosine diphosphate; PEP = Phosphoenolpyruvate; PK = Pyruvate kinase; LDH = Lactate dehydrogenase; NADH = Nicotinamide adenine dinucleotide (reduced); NAD^+ = Nicotinamide adenine dinucleotide (oxidised). Reaction 1 represents the production of ADP from glucuronic acid and ATP by glucuronokinase. Reaction 2 represents the oxidation of NADH reaction initiated by the addition of ADP produced in reaction 1.

3.2.7. Statistical analysis

Data was analysed for variance (1-way ANOVA) using the Daniel's XL Toolkit (v 6.60, Free Software Foundation Inc., Boston, USA). A modified version of the Levene's test described by Glantz and Slinker (2001) was first used to determine equal variance among sugar concentrations among transgenic lines. Secondly, the interaction between and within the sugar concentrations of each transgenic line was tested for significance ($p \leq 0.05$). Lastly, significance between sugar content amongst each transgenic line was determined by the least significant difference (LSD) using the Bonferroni-Holm Post hoc test (Daniels XL Toolbox, v 6.60, Free Software Foundation Inc., Boston, USA).

3.3   Results

3.3.1. Determining gene confirmation and expression in putatively transformed NCo310 plants

3.3.1.1 Confirmation of transgene insert

Genomic DNA extracted from acclimatised putatively transformed plant leaves were used to confirm transgene insertion using both gene specific and gene specific (forward) combined with CamV terminator (reverse) primers. Gene insertion was confirmed in all 10 putative lines, using both primer pair sets (Figure 3.5).
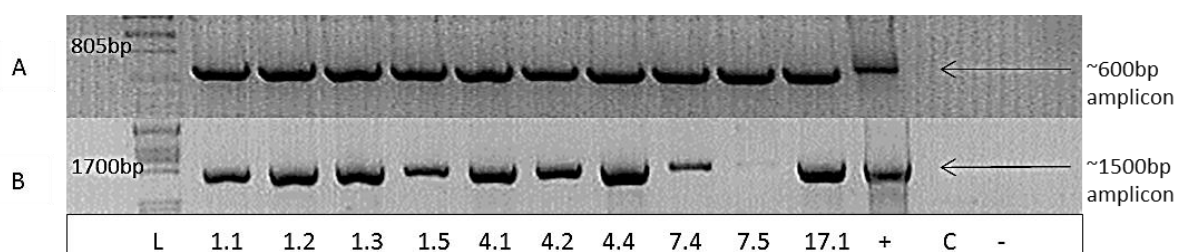

Figure 3.5:   PCR amplification of transgenes from genomic DNA extracted from putatively transformed sugarcane plantlets viewed on a 1.5% (w/v) agarose gel. Gene amplification using gene specific primers (A) and a combination of gene specific forward primer and the CamV reverse primer (B). L indicates the Pst lambda DNA marker; + indicates the positive vector DNA (pUBI 510 :: *Gluc*); C indicates the wildtype sugarcane control; and – indicates the negative water control. Arrows indicate the estimated band size of the amplicon.

3.3.1.2 Transgene expression analyses

SqRT-PCR amplification was used to determine the presence and relative expression levels of the inserted gene in the different transgenic plant lines. The expression of the actin housekeeping gene (*ACTIN 2*) was used as an internal standard (Figure 3.6). Results showed that all, but lines 4.2 and 7.5 expressed the glucuronokinase transgene on RNA level. These lines were therefore excluded from further experiments. Expression levels however differ between lines, varying from high expression in lines 1.1, 1.5, 4.4, 7.4 and 17.1, and low expression in line 1.3 (Figure 3.6).
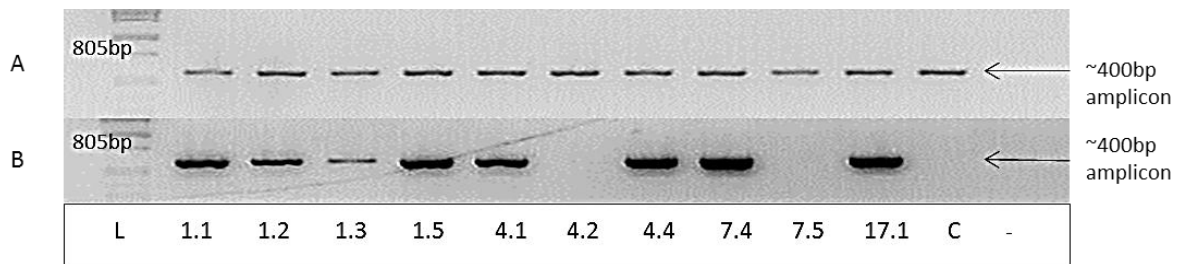
Figure 3.6:   SqRT-PCR amplification analysis using cDNA template obtained from putatively transformed plantlet leaves with actin (A) and gene specific (B) primers viewed on a 2% (w/v) agarose gel. L indicates Pst lambda DNA marker; C indicates the wildtype sugarcane control; and – indicates the negative water control. Arrows indicate the estimated band size of the amplicon.

3.3.2. Determining phenotypic variations in glucuronokinase overexpressing NCo310 lines

Various phenotypes were observed among the transgenic lines compared to the non-transgenic control plants (Figure 3.7). Out of the eight transgenic expressing lines, four displayed a grass-like bushy phenotype that did not form any mature stalks, which were lines 1.1, 1.2, 1.3 and 1.5 (Figure 3.7 A). The transgene expression profiles varied between these lines, ranging from low expression in line 1.3 to high expression in line 1.5 (Figure 3.7). The remaining four lines, 4.1, 4.4, 7.4 and 17.1 were able to form maturing stalks similar to that seen in the non-transgenic control plant (Figure 3.7 B). These transgenic plants' stalks however, seemed to be not as thick as the stalks formed by the control plant (Figure 3.7 B). Transgene mRNA accumulation in the stalk-forming transgenic plants were all relative high (Figure 3.6).

Figure 3.7: Phenotypic variations of glucuronokinase overexpressing lines grown in a growth tunnel at the Stellenbosch Welgevallen experimental farm. Lane A represents transgenic lines demonstrating the bushy phenotype and lane B represents transgenic lines demonstrating the stalk-forming phenotype, comparable to that seen in the NCo310 wildtype control. Arrow indicates maturing stalks.

3.3.3. Determining glucuronokinase enzyme activity in NCo310 transgenic lines

Crude protein extracts were isolated from leaf material and used to assay glucoronokinase activity in both continuous and stopped assays.

3.3.3.1 Continuous assay

In the continuous assay, there was an unexpected background NADH oxidase activity that masked glucuronokinase activity (data not shown). To overcome the unexpected NADH oxidation, a stopped assay was conducted.

3.3.3.2 Stopped assay

A stopped assay would overcome the background NADH oxidation as the NADH oxidase activity would be denatured prior to testing of ADP production (Figure 3.4).

Enzyme activity was expressed as μmol NADH oxidised per minute per mg total protein. ADP was used as a positive control (data not shown). Although slight activity was observed, no significant differences in activity were observed between the glucuronokinase transgenic lines and the NCo310 control (Figure 3.8). Furthermore, no link between the transgene expression levels and the enzyme activity levels could be formulated. The only observation was that the lowest expressing transgenic line, namely line 1.3, displayed the lowest enzymatic activity when compared to other transgenic lines and the NCo310 control.
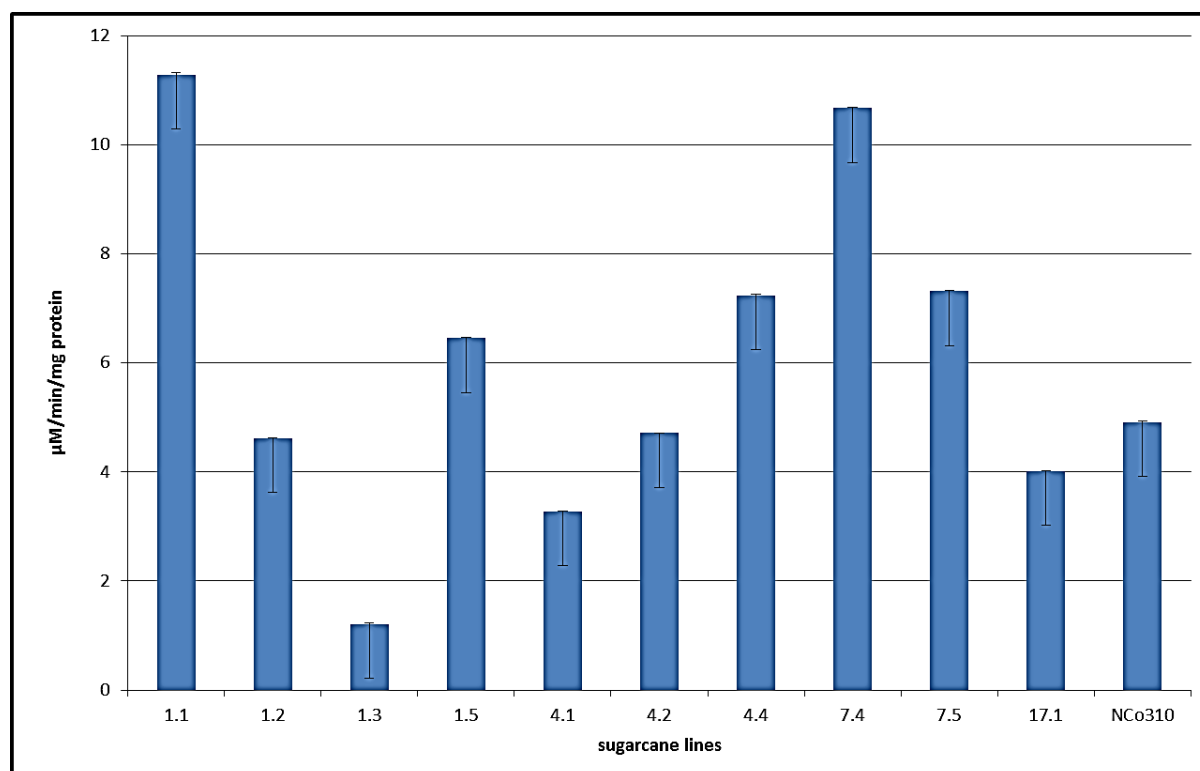

Figure 3.8: A graph illustrating glucuronokinase enzyme activity in sugarcane leaves of transgenic lines. Significant difference was measured at $p < 0.05$ (No indicators demonstrate no significant differences between transgenic lines). NCo310 represents the wildtype sugarcane control. Error bars represent the standard error of the mean n= 3.

3.3.4. Determining sugar content in glucuronokinase overexpressing NCo310 lines

3.3.4.1 Soluble sugar concentrations

Sugars were determined from leaf material (Figure 3.9). Results indicated very low levels of glucose and fructose in the transgenic leaves (data not shown). There were no significant differences in the sucrose content across all tested lines compared to the NCo310 control line (Figure 3.9).
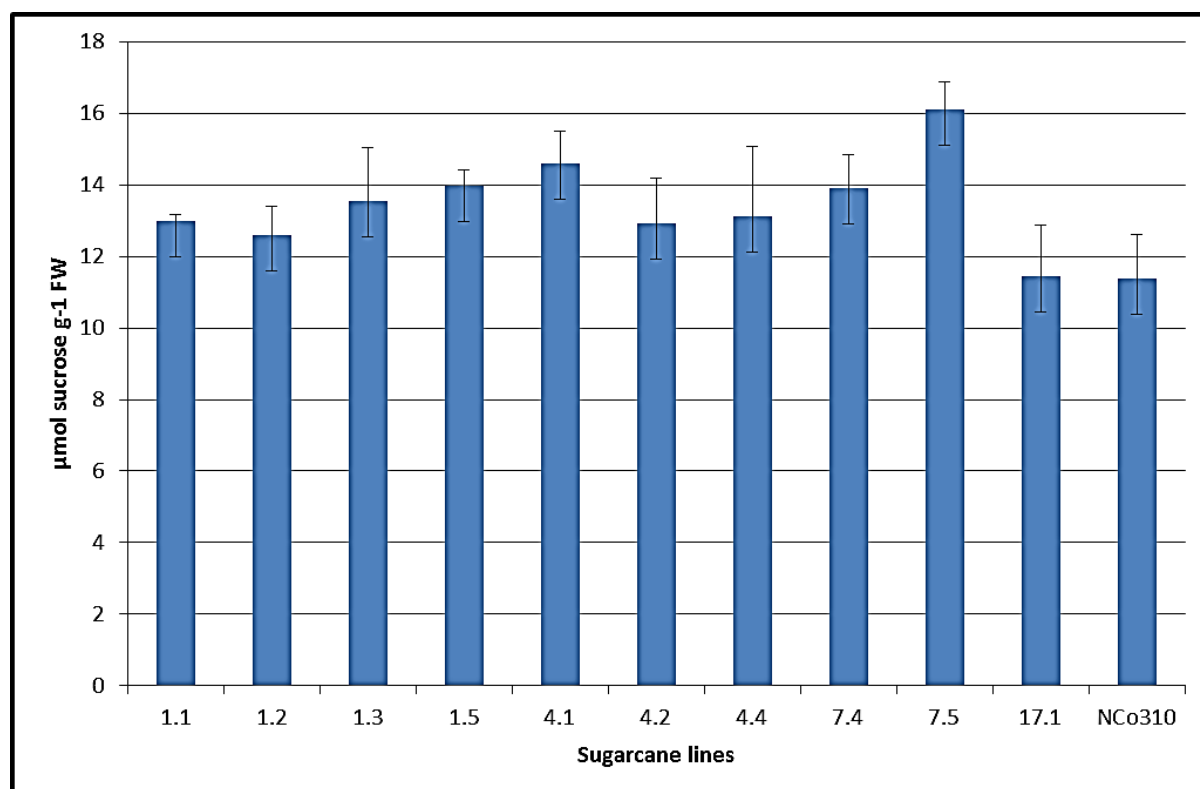
Figure 3.9: Analysis of sucrose concentrations present in sugarcane leaves of glucuronokinase expressing lines. Each transgenic line represents a mean value calculated from three biological repeats with technical triplicates. NCo310 represents the wildtype sugarcane control. Significant difference was measured at $p < 0.05$ (No indicators demonstrate no significant differences between transgenic lines).

3.4 Discussion

3.4.1 Glucuronokinase transformation and expression in sugarcane lines

The NCo310 sugarcane cultivar was successfully transformed with the glucuronokinase gene isolated from Arabidopsis (Figure 3.5). With the exception of two lines, all the lines showed relatively high expression levels at RNA level (Figure 3.6 B). High gene expression at RNA level does not, however, necessarily correlate with the enzyme activity (Figure 3.8). In this study glucuronokinase activities were not significant different between the control and transgenic lines. However, activity was only measured in leaf samples and might differ in the various tissue types within the sugarcane plant. Furthermore, it is generally more difficult to measure enzyme activity in leaf material than in internodes. Dickinson (1982) was able to detect glucoronokinase activity in tissue such as leaves, callus, pollen and seedlings from various plant species, with pollen showing the highest activity. Enzyme activity in these tissues were however much lower, at nmol/min/mg protein levels as measured at the time by radioactive isotope labelling. In a more recent study, the glucoronokinase enzyme has been purified from only Easter lily (*Lilium longiflorum*) pollen (Pieslinger *et al.*, 2009). Furthermore, Pieslinger and colleagues were able to isolate the glucoronokinase gene from *Arabidopsis thaliana* flowers, but the purified recombinant protein was then expressed and analysed in bacteria and not *in situ*. Overall, limited knowledge is available to serve as reference point to compare the measured enzyme activity levels present in crude sugarcane extracts. It might be useful in the future to also employ other techniques such as high performance liquid chromatography (HPLC) to measure the glucoronokinase activity.

As shown in Figure 3.7, some transgenic plants grown in the pot trial displayed phenotypes different from the control non-transgenic plants. Two distinct phenotypes were seen; the first displayed a grass-like, bushy phenotype in four of the transgenic lines that did not form any mature stalks over a twelve month period.   Secondly, other transgenic lines formed elongating mature stalks but these were thinner than those observed in the wildtype control. One hypothesis is that glucoronokinase is possibly involved in sugar signalling. In turn sugar signalling may influence hormonal signalling in plants resulting in changes in growth and development (Léon and Sheen, 2003). Connections between sugar signalling and hormone pathways have been well studied in the past (Matsoukas, 2014). For example, hexokinase is a glucose sensor in plants and has been shown to integrate light, hormones and nutrient signalling to control plant growth (Rolland and Sheen, 2005).

3.4.2 Sugar content and enzymatic activity

No significant differences in sugar content were seen in the transgenic plants when compared to the control plants (Figure 3.9). However, these measurements were only done on leaf material and not mature sugarcane stalks. I hypothesise that overexpression of the

glucoronokinase gene can lead to two possible outcomes. Firstly, it can lead to a flux in sugar movement towards the cell wall which, potentially can lead to less sucrose in the cane samples.   Secondly, overexpression of glucoronokinase, acting as a sugar signalling molecule, can lead to a decrease in sugar flux and either an increase or a decrease in cane sucrose. Either one of these hypotheses are possibly true, however will only be confirmed when the soluble sugar content and cell wall composition are determined in the transgenic mature stalk material in the future.

3.4.3 Future improvements

For future considerations:

Plant cell wall components, lignocellulosic biomass as well as sugars are produced from UDP-D-glucuronic acid (UDP-Glc A), a precursor produced by two pathways, the sugar nucleotide oxidation pathway (SNOP) and the myo-inositol oxidation pathway (MIOP) (Loewus and Murthy, 2000; Loewus *et al.*, 1962; Seifert, 2004). It has not yet been clarified as to which pathway is the predominant one used in plants to produce cell wall components. However, it has been shown that the presence and/or absence of either inositol or glucose, due to factors such as developmental stages, can result in either pathway dominating. Work done by Bekker (2007) demonstrated increase of sucrose in SNOP compromised sugarcane lines, possibly due to the increased activity of enzymes involved in the MIOP. The fact that this study was a based on Bekker's (2007) results, but did not use SNOP compromised (UGD repressed) sugarcane lines to mimic the results obtained by Bekker (2007), might have rendered the use of the *myo*-inositol oxidation pathway negligible and sugars could still be synthesised by the sugar nucleotide oxidation pathway. Therefore, the most prominent issues that should be considered or recommended in future for this study include; i) using plants with a compromised SNOP such as repressed UGD enzyme activity, which might be a possible signal to produce cell wall sugars and other components using the *myo*-inositol pathway (Bekker, 2007; Labate *et al.*, 2008; Leowus and Leowus, 1980; Seitz *et al.*, 2000) and ii) explore any cell wall components, sugars or lignocellulosic biomass, an important feature that should be investigated if the study is to help produce plants viable for biofuel production. Furthermore, in the future, the current use of only leaf material to investigate sucrose content in transgenic plants should be extended to include the use of culm tissue (young, intermediate and mature) to obtain a complete picture regarding sucrose content in these transgenic plants.

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Chapter 4

General summary and future considerations

In South Africa, agronomic drought poses a serious threat that has accounted for drastic crop productivity and yield diminution in recent years. Furthermore, the use of fossil fuels as the primary source of energy has resulted in rather deleterious effects on the sustainability of the environment. This issue has led to research efforts that aim to counteract these negative effects in order to protect and sustain the environment. In this regard, research has managed to introduce the possibility of plants as replacements of fossil fuels in terms of being the main source of energy. Sugarcane is the primary crop in the South African sugar industry and contributes largely towards the country's economy. It is also a crop that research has shown to have great potential as a biofuel source. This project was tasked to apply biotechnological approaches to unravel and improve the potential of sugarcane as a food and biofuel source.

The first part of the project applied chemical mutagenesis followed by *in vitro* osmotic tolerance selection on sugarcane callus. This study was conducted to assist in combating the effects of drought, e.g. deleterious effects on crop productivity. Calli from three sugarcane cultivars (N19, NCo376 and NCo310), of which two (N19 and NCo376) are reported by the South African Sugarcane Research Institute (SASRI) to be susceptible to drought in the field were used. Calli cells were EMS treated with 16 mM ethylmethane sulfonate (EMS) and selected for osmotic tolerance on nutrient media supplemented with polyethylene glycol (PEG) *in vitro.* The selection was comprised of three phases (8 weeks in the dark on PEG; 2 week recovery in light without PEG; 8 weeks in light on PEG), which exposed callus cells to PEG for long periods to allow for efficient selection. From all cultivars, clones were identified that survived the *in vitro* PEG selection and these were prepared for *ex vitro* drought pot trials. Due to time restraints only selected NCo310 clones were exposed to *ex vitro* drought pot trials to further confirm their potential enhanced drought tolerance. Out of 19 clones that showed *in vitro* PEG tolerance, only six demonstrated improved drought tolerance during the drought pot trials. Five of these six lines outlived the drought tolerant NCo310 wildtype control (SASRI communications), only one line demonstrated recovery upon re-watering after plant death.

In the future comprehensive experimental tests will need to be conducted in order to assess the morphology and confirm drought tolerance in these selected mutant lines. The first experiment will have to look at the mutant plants under non-stress conditions and see if they

differ from the wildtype control in shape, size and other traits such as sucrose content. The observed differences between the mutated and osmoticum selected clones deviation as well as the wildtype control might potentially influence and explain plant performance under stress conditions. Under drought stress conditions changes in traits such as RWC (relative water content), photosynthesis, turgor maintenance, stomatal closure, transpiration, antioxidant activity as well as shoot and root architecture might be evaluated to complete analysis of these mutant lines. Furthermore, molecular techniques such as gene expression profile analysis might be employed to provide additional understanding of the molecular basis of the obtained trait. Also, to attain a complete picture on the drought tolerant abilities of these mutant lines, analysis will have to be conducted at appropriate growth phases (germination, tillering, grand growth or ripening phases) within the live cycle of the sugarcane plant.

The second part of the project was based on a study conducted by Bekker (2007), where UDP-glucose dehydrogenase, an enzyme in the sugar nucleotide oxidation pathway (SNOP), was downregulated in NCo310 sugarcane. Those UGD repressed sugarcane lines showed an unexpected increase in sucrose and cell wall cellulose content. Changes in this pathway could, therefore, lead to plants with potentially increased sucrose and alterations in cell wall components that may be useful for biofuel production. These unexpected findings led to a hypothesis that when the SNOP is downregulated, the *myo*-inositol oxidation pathway (MIOP) assumes the function of producing UDP-glucuronic acid, a precursor of cell wall sugars and lignocellulosic biopolymers. To further investigate this hypothesis, Bekker (2007) determined the expression of the first enzyme in the MIOP, the *myo*-inositol oxygenase enzyme (MIOX), in transgenic sugarcane and found that it the MIOX transcript demonstrated a significant increase in expression. It was therefore important to research the MIOP further in order to understand its function in cell wall biosynthesis and sucrose metabolism in sugarcane.

The second part of this project was therefore tasked to build on the Bekker's (2007) study and aimed to upregulate the glucuronokinase gene, which encodes the second enzyme in the MIOP, in sugarcane to determine if there will be any alterations in sucrose content in the transgenic plants.

NCo310 transgenic sugarcane lines that overexpressed the glucuronokinase gene did not show any significant differences in both sucrose content and glucoronokinase enzymatic activity. However, phenotypic differences were observed between transgenic lines and wildtype sugarcane. Half of the transgenic lines displayed a glass-like bushy phenotype that did not form stalks. The remainder developed mature stalks similar to those observed in the

wildtype control plants, however, these stalks were not as thick as those formed in the wildtype control plants. When correlating the expression pattern of the transgene with the observed phenotypes, plants that demonstrated the grassy bushy phenotype had inconsistent transgene expression profiles ranging from low to high expression. Transgenic plants that were able to form stalks had relatively high expression of the transgene.

The lack of research regarding the correlation between the MIOP and its enzymes to sucrose metabolism impedes comparisons or references to make concrete conclusions from findings in this study. In the future in might also be useful to investigate possible alterations in cell wall components, such as cellulose and cell wall sugars to complete the analysis of these glucoronokinase transgenic plants. In addition, to improve on the current study in might be useful to revisit the UGD repressed sugarcane lines to explore in more detail the MIOP and its enzymes in sucrose metabolism and cell wall biosynthesis and determine alterations in abundance of cell wall components such as cellulose and cell wall sugars.